APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE : Methods For Altering Cell Fate

METHODS FOR ALTERING CELL FATE

Cross-Reference to Related Application

This application claims the benefit of the filing date of U.S. provisional application 60/258,152, filed December 22, 2000.

Background of the Invention

In general, the invention features methods for converting cells into a desired cell type and methods for administering these reprogrammed cells to a mammal for the treatment or prevention of disease.

Despite having essentially the same genome, different classes of somatic cells in a particular mammal have distinctive phenotypes due to the different combinations of genes that they express. These different expression profiles allow cells to perform certain functions, such as the secretion of a hormone or cartilage.

Because many diseases and injuries are caused by damage to a particular class of cells, methods are needed to produce cells of a desired cell type that may be used to replace these damaged cells. Preferably, these replacement cells have the same genotype as the damaged cells.

Summary of the Invention

The purpose of the present invention is to provide methods for altering the characteristics or functions of cells. In particular, these methods involve incubating a nucleus or chromatin mass from a donor cell with a reprogramming media (e.g., a cell extract) under conditions that allow nuclear or cytoplasmic components such as transcription factors to be added to, or removed from, the nucleus or chromatin mass.

Preferably, the added transcription factors promote the expression of mRNA or protein molecules found in cells of the desired cell type, and the removal of transcription factors that would otherwise promote expression of mRNA or protein molecules found in the donor cell. If desired, the chromatin mass may then be incubated in an interphase reprogramming media (*e.g.*, an interphase cell extract) to reform a nucleus that incorporates desired factors from either reprogramming media. Then, the nucleus or chromatin mass is inserted into a recipient cell or cytoplast, forming a reprogrammed cell of the desired cell type. In a related method, a permeabilized cell is incubated with a reprogramming media (*e.g.*, a cell extract) to allow the addition or removal of factors from the cell, and then the plasma membrane of the permeabilized cell is resealed to enclose the desired factors and restore the membrane integrity of the cell. If desired, the steps of any of these methods may be repeated one or more times or different reprogramming methods may be performed sequentially to increase the extent of reprogramming, resulting in a greater alteration of the mRNA and protein expression profile in the reprogrammed cell. Furthermore, reprogramming medias may be made representing combinations of cell functions (*e.g.*, medias containing extracts or factors from multiple cell types) to produce unique reprogrammed cells possessing characteristics of multiple cell types.

Accordingly, in a first aspect, the invention provides a method of reprogramming a cell. This method involves incubating a nucleus with a reprogramming media (*e.g.*, a cell extract) under conditions that allow the removal of a factor from the nucleus or the addition of a factor to the nucleus. Then the nucleus or a chromatin mass formed from incubation of the nucleus in the reprogramming media is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. In one preferred embodiment, the nucleus is incubated with an interphase reprogramming media (*e.g.*, an interphase cell extract). Preferably, the nucleus remains membrane-bounded, and the chromosomes in the nucleus do not condense during incubation with this interphase reprogramming media. In another preferred embodiment, a chromatin mass is formed from incubation of the nucleus in a mitotic reprogramming media (*e.g.*, a mitotic extract). Preferably, this

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chromatin mass is then incubated in an interphase reprogramming media under conditions that allow a nucleus to reform, and the reformed nucleus is inserted into the recipient cell or cytoplast.

In a related aspect, the invention provides another method of reprogramming a cell. This method involves incubating a chromatin mass with a reprogramming media (*e.g.*, a cell extract) under conditions that allow the removal of a factor from the chromatin mass or the addition of a factor to the chromatin mass. Then the chromatin mass or nucleus formed from incubation of the chromatin mass in a reprogramming media (*e.g.*, an interphase extract) is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. In one preferred embodiment, the chromatin mass is generated by incubating a nucleus from a donor cell in a detergent and salt solution, in a protein kinase solution, or in a mitotic reprogramming media in the presence or absence of an antibody to NuMA or to another protein of the nucleus. In another preferred embodiment, the chromatin mass is isolated from mitotic cells.

In another related aspect, the invention provides yet another method of reprogramming a cell. This method involves incubating a permeabilized cell with a reprogramming media (*e.g.*, a cell extract) under conditions that allow the removal of a factor from the nucleus or chromatin mass of the permeabilized cell or the addition of a factor to the nucleus or chromatin mass, thereby forming a reprogrammed cell. In one preferred embodiment, the permeabilized cell is incubated with an interphase reprogramming media (*e.g.*, an interphase cell extract). Preferably, the nucleus in the permeabilized cell remains membrane-bounded, and the chromosomes in the nucleus do not condense during incubation with this interphase reprogramming media. In another preferred embodiment, a chromatin mass is formed from incubation of the permeabilized cell in a mitotic reprogramming media. In yet another preferred embodiment, the reprogrammed cell is incubated under conditions that allow the membrane of the reprogrammed cell to reseal. If desired, the permeabilized cell may be formed by incubating an intact cell with a detergent, such as digitonin, or a bacterial toxin, such as Streptolysin O.

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The invention also provides reprogrammed cells generated using any method of the invention or a combination of methods of the invention. These cells are useful for the treatment or prevention of a disease due to a deficiency in a particular cell type. Additionally, reprogrammed cells that express two or more mRNA molecules or proteins

5   that are each specific for a certain cell type may have novel combinations of phenotypes and activities that are useful for the treatment of disease. For example, cells that maintain the ability of the donor cell to divide and gain the ability to form a functional T-cell receptor or a functional neurofilament are useful for the generation of multiple T-cells or neurons for therapeutic applications. Once transplanted into a subject, these cells may

10   maintain the ability to divide, thereby reducing the dose or dosing frequency of the transplant cells that is required to treat, prevent, or stabilize a disease. The characterization of these cells may also result in the identification of proteins involved in the regulation of gene expression.

In one such aspect, the invention features a cell that expresses a combination of

15   two or more endogenous mRNA molecules or endogenous proteins that is not expressed by a naturally-occurring cell. In a related aspect, the invention features a cell that expresses a combination of two or more endogenous mRNA molecules or endogenous proteins at a level that is at least 10, 20, 50, 75, or 100 fold greater than the expression level of the corresponding mRNA molecules or proteins in any naturally-occurring cell.

20   In preferred embodiments of the above aspects, the cell expresses a combination of 5, 10, 25, 50, 75, 100, 150, 300, or more endogenous mRNA molecules or endogenous proteins that is not expressed by a naturally-occurring cell. In another preferred embodiment, the cell expresses 1, 3, 5, 10, 25, 50, 100, or more endogenous mRNA molecules or endogenous proteins that are specific for one cell type and expresses 1, 3, 5, 10, 25, 50,

25   100, or more endogenous mRNA molecules or endogenous proteins that are specific for another cell type. In other preferred embodiments, the cell has a combination of 2, 5, 10, 25, 50, 75, 100, 150, 300, or more activities or phenotypes that are not exhibited in a naturally-occurring. In yet other preferred embodiments, the cell is able to divide or is immortalized and expresses a neuronal protein such as the NF200 neurofilament protein

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or any other protein expressed by differentiated neurons. In still other preferred embodiments, the cell is able to divide or is immortalized and expresses IL-2, an IL-2 receptor, a T-cell receptor, CD3, CD4 and CD8, CD45 tyrosine phosphatase, or any other protein expressed in hematopoietic cells. In yet another embodiment, the cell is formed

5   from the reprogramming of a donor fibroblast cell, nucleus, or chromatin mass, and the reprogrammed cell expresses one or more cytoskeleton proteins such as an integrin at a level that is at least 25, 50, 75, 90, or 95% lower that the corresponding level in the donor fibroblast under the same conditions. In another embodiment, a reprogrammed cell formed from a donor fibroblast or liver cell (*e.g.*, a hepatocyte) expresses IL-2, a

10   neurofilament protein, a T-cell receptor, Oct4, or insulin.

In a related aspect, the invention provides a cell that expresses a T-cell specific protein (*e.g.*, T-cell receptor protein, IL-2 receptor, CD3, CD4, or CD8) and one or more fibroblast-specific proteins. Preferably, stimulation of the cell with an antigen or an anti-CD3 antibody induces the expression of the α-chain of the IL-2 receptor. In another

15   aspect, the invention provides a cell that expresses a hematopoietic-specific protein (*e.g.*, CD45 typrosine phosphatase) and one or more fibroblast-specific proteins. In another related aspect, the invention provides a cell that expresses a neuron-specific protein (*e.g.*, a neurofilament protein such as NF200) or forms neurites and expresses one or more fibroblast-specific proteins. In still another aspect, the invention provides a cell that

20   expresses a neurofilament protein (*e.g.*, NF200) or forms neurites and is immortalized. In yet another aspect, the invention provides a cell that expresses a stem cell-specific protein (*e.g.*, Oct4) or alkaline phosphatase and one or more fibroblast-specific proteins. In still another aspect, the invention provides a cell that expresses one or more fibroblast-specific proteins and grows in aggregates, forms colonies, or forms embryoid bodies.

25   Preferred fibroblast-specific proteins include cell adhesion molecules that *e.g.*, promote anchoring of one or more reprogrammed cells to a site of interest in a host patient. Fibroblast-specific growth factors (*e.g.*, the FGF family of proteins) are other exemplary fibroblast-specific proteins.

These methods for reprogramming cells are useful for the generation of cells of a desired cell type, for example, for medical applications. Accordingly, the invention also provides methods for the treatment or prevention of disease in a mammal that include administering a reprogrammed cell to the mammal.

5   In one such method, the invention features a procedure for treating or preventing a disease, disorder, or condition in a mammal. This method involves incubating a nucleus from a donor cell with a reprogramming media (*e.g.*, a cell extract) under conditions that allow the removal of a factor from the nucleus or the addition of a factor to the nucleus. The nucleus or a chromatin mass formed from the nucleus is inserted into a recipient cell

10   or cytoplast, thereby forming a reprogrammed cell. The reprogrammed cell is then administered to the mammal in need of the cell type. In one preferred embodiment, the nucleus is incubated with an interphase reprogramming media. Preferably, the nucleus remains membrane-bounded, and the chromosomes in the nucleus do not condense during incubation with this interphase reprogramming media. In another preferred

15   embodiment, a chromatin mass is formed from incubation of the nucleus in a mitotic reprogramming media. Preferably, this chromatin mass is then incubated in an interphase reprogramming media under conditions that allow a nucleus to be formed from the chromatin mass, and the reformed nucleus is inserted into the recipient cell or cytoplast. Preferably, the donor cell is from the mammal (for example, a human) in need of the cell

20   type. Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for

25   reconstructive applications, such as for repairing or replacing tissues or organs.

In a related aspect, the invention provides another method of treating or preventing a disease, disorder, or condition in a mammal (for example, a human). This method involves incubating a chromatin mass from a donor cell with a reprogramming media (*e.g.*, a cell extract) under conditions that allow the removal of a factor from the

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chromatin mass or the addition of a factor to the chromatin mass. The chromatin mass or a nucleus formed from incubating the chromatin mass in an interphase reprogramming media is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. In one preferred embodiment, the chromatin mass used in this method is generated by

5   incubating a nucleus from a donor cell in a detergent and salt solution, in a protein kinase solution, or in a mitotic reprogramming media in the presence or absence of an antibody to NuMA. In another preferred embodiment, the chromatin mass is isolated from mitotic cells. The reprogrammed cell is then administered to a mammal in need of the cell type. Preferably, the donor cell is from the recipient mammal. Examples of diseases, disorders,

10   or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or

15   replacing tissues or organs.

In still another related aspect, the invention provides another method of treating or preventing a disease, disorder, or condition in a mammal (for example, a human) that involves incubating a permeabilized cell with a reprogramming media (*e.g.*, a cell extract) under conditions that allow the removal of a factor from the nucleus or chromatin

20   mass of the permeabilized cell or the addition of a factor to the nucleus or chromatin mass. The reprogrammed cell formed from this step is administered to a mammal in need of that cell type. In one preferred embodiment, the permeabilized cell is incubated with an interphase reprogramming media. Preferably, the nucleus in the permeabilized cell remains membrane-bounded, and the chromosomes in the nucleus do not condense

25   during incubation with the interphase reprogramming media. In another preferred embodiment, a chromatin mass is formed from incubation of the permeabilized cell in a mitotic reprogramming media. In yet another preferred embodiment, the reprogrammed cell is incubated under conditions that allow the membrane of the reprogrammed cell to reseal prior to being administered to the mammal. Preferably, the permeabilized cell is

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from the mammal in need of that cell type. In another preferred embodiment, the permeabilized cell is formed by incubating an intact cell with a detergent, such as digitonin, or a bacterial toxin, such as Streptolysin O. Examples of diseases, disorders, or conditions that may be treated or prevented include neurological, endocrine, structural, skeletal, vascular, urinary, digestive, integumentary, blood, immune, auto-immune, inflammatory, endocrine, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

The invention also provides methods for measuring an endogenous activity (*e.g.*, an endogenous enzymatic activity) or an endogenous protein in a cell, nucleus, chromatin mass, cell lysate, or *in vitro* sample. In one such aspect, the method involves contacting a solid support with a test sample from a cell, nucleus, chromatin mass, cell lysate, or *in vitro* sample, and with a reference sample. The test sample has an endogenous activity of interest that is naturally found in the test sample (*e.g.*, luciferase activity or phosphatase activity, *e.g.*, alkaline phosphatase activity), and the test sample has a known protein concentration or is derived from a known number of cells. The reference sample has a known level of the activity of interest (*e.g.*, luciferase activity or phosphatase activity) or a known amount of naturally-occurring or recombinant protein having the activity. The level of luciferase or phosphatase activity in the test sample is measured and compared to the level of luciferase or phosphatase activity in the reference sample, thereby determining the level of luciferase or phosphatase activity in the cell, nucleus, chromatin mass, cell lysate, or *in vitro* sample. In one preferred embodiment, the luciferase or phosphatase activity is performed by a naturally-occurring protein encoded by an endogenous nucleic acid under the control of an endogenous promoter. This method may also be used to measure any other endogenous activity of interest. In another preferred embodiment, the activity is specific for one cell type or specific for a family of related cell types. In various embodiments, the activity of interest is the chemical alteration of one or more substrates to form a product. Preferably, either one of the substrates or one

of the products is detectable. Detectable labels are well known in the art and include, without limitation, radioactive labels (*e.g.*, isotopes such as ^{32}P or ^{35}S) and nonradioactive labels (*e.g.*, chemiluminescent labels or fluorescent labels, *e.g.*, fluorescein).

In a related aspect, the invention provides a method for measuring the level of an

5 endogenous protein in a cell, nucleus, chromatin mass, cell lysate, or *in vitro* sample. This method involves contacting a solid support with a test sample from a cell, nucleus, chromatin mass, cell lysate, or *in vitro* sample, and with a reference sample. The test sample has an endogenous, detectable protein of interest (*e.g.*, luciferase, alkaline phosphatase, or Oct4) that is naturally found in the test sample, and the test sample has a

10 known protein concentration or is derived from a known number of cells. The reference sample has a known amount of the protein of interest, *e.g.*, naturally-occurring or recombinant luciferase, alkaline phosphatase, or Oct4 protein. The signal from the luciferase, alkaline phosphatase, or Oct4 protein in the test sample is measured and compared to the signal from the corresponding protein in the reference sample, thereby

15 determining the amount of luciferase, alkaline phosphatase, or Oct4 protein in the cell, nucleus, chromatin mass, cell lysate, or *in vitro* sample. This method may also be used to measure the level of any other endogenous protein of interest. In one preferred embodiment, the protein of interest is encoded by an endogenous nucleic acid under the control of an endogenous promoter. In a preferred embodiment, the protein of interest is

20 specific for one cell type or specific for a family of related cell types. In various embodiments, the protein of interest has a detectable label or binds another molecule (*e.g.*, an antibody) with a detectable label. Exemplary detectable labels include radioactive labels (*e.g.*, isotopes such as ^{32}P or ^{35}S) and nonradioactive labels (*e.g.*, chemiluminescent labels or fluorescent labels, *e.g.*, fluorescein).

25 In preferred embodiments for the above methods of measuring the level of an activity or protein of interest, the solid support is contacted with multiple reference samples, each with a different level of activity or a different amount of the protein of interest. According to this embodiment, a standard curve may be generated from the reference samples and used to determine the level of activity or the amount of the protein

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of interest in the test sample. In various embodiments, the cell is a stem cell such as an embryonic stem cell or an adult stem cell from brain, blood, bone marrow, pancreas, liver, skin, or any other organ or tissue. In other embodiments, the cell has been exposed to an extract from a stem cell. In yet other embodiments, the test sample is a from a

5   nuclear or cytoplasmic cell extract. Useful solid supports include any rigid or semi-rigid surface that may be contacted with the sample. The support can be any porous or non-porous water insoluble material, including, without limitation, membranes, filters, chips, slides, fibers, beads, gels, tubing, strips, plates, rods, polymers, particles, microparticles, capillaries, and plastic surfaces. If desired, the support can have a variety of surface

10   forms, such as wells, trenches, pins, channels and pores, to which the samples are contacted.

In preferred embodiments of various aspects of the invention, at least 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 300, or more mRNA or protein molecules are expressed in the reprogrammed cell that are not expressed in the donor or permeabilized cell. In

15   another preferred embodiment, the number of mRNA or protein molecules that are expressed in the reprogrammed cell, but not expressed in the donor or permeabilized cell, is between 1 and 5, 5 and 10, 10 and 25, 25 and 50, 50 and 75, 75 and 100, 100 and 150, 150 and 200, or 200 and 300, inclusive. Preferably, at least 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 300, or more mRNA or protein molecules are expressed in the donor or

20   permeabilized cell that are not expressed in the reprogrammed cell. In yet another preferred embodiment, the number of mRNA or protein molecules that are expressed in the donor or permeabilized cell, but not expressed in the reprogrammed cell, is between 1 and 5, 5 and 10, 10 and 25, 25 and 50, 50 and 75, 75 and 100, 100 and 150, 150 and 200, or 200 and 300, inclusive. Preferably, the mRNA or protein molecules are specific for

25   the cell type of the donor, permeabilized, or reprogrammed cell, such that the molecules are only expressed in cells of that particular cell type. In still another preferred embodiment, these mRNA or protein molecules are expressed in both the donor cell (*i.e.*, the donor or permeabilized starting cell) and the reprogrammed cell, but the expression levels in these cells differ by at least 2, 5, 10, or 20-fold, as measured using standard

assays (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000). In other embodiments, the expression of one or more cytoskeleton proteins such as integrins is decreased by at least at least 2, 5, 10, or 20-fold compared to the donor fibroblast cell. In yet other embodiments, the reprogrammed cell expresses a neurofilament protein, T-cell receptor protein, IL-2, IL-2 receptor, insulin, or Oct4 at a level that is at least 2, 5, 10, or 20-fold greater that the corresponding level in the donor or permeabilized cell.

In other preferred embodiments, the size of the donor or permeabilized cell differs from that of the reprogrammed cell by at least 10, 20, 30, 50, 75, or 100%, as measured using standard methods. In another preferred embodiment, the volume of cytoplasm in the donor or permeabilized cell differs from that in the reprogrammed cell by at least 10, 20, 30, 50, 75, or 100%, based on standard methods. In yet another preferred embodiment, the reprogrammed cell has gained or lost an activity relative to the donor or permeabilized cell, such as secretion of a particular hormone, extracellular matrix component, or antibody. In another embodiment, the reprogrammed cell has gained the ability to produce and secrete an interleukin such as IL-2, gained the ability to form a neurofilament, neurite, or axon, or gained the ability to form an embryoid body. In another embodiment, cell has gained the ability to express a T-cell receptor or IL-2 receptor or to produce insulin. Preferably, the β-chain of the IL-2 receptor is expressed constitutively, and the α-chain is expressed upon activation (*e.g.*, by stimulation with an anti-CD3 antibody or by presentation of an antigen). In other embodiments, a reprogrammed cell such as a stem cell or fibroblast has gained the ability to contract, resembling a contracting muscle cell or beating heart cell.

In still other preferred embodiments, the reprogramming media is an interphase reprogramming media, such as an extract formed from cells synchronized in one or more of the following phases of the cell cycle: G_o, G_1, S, or G_2 phase. In another preferred embodiment, the reprogramming media is an extract formed from cells synchronized in mitosis or from unsynchronized cells. Preferably, the reprogramming media is an extract from the cell type one wishes the donor or permeabilized cell to become, or the

reprogramming media is a solution containing factors specific for the cell type one wishes the donor or permeabilized cell to become. Examples of cells that may be used to generate extracts to reprogram cells into stem cells include embryonic stem cells and adult stem cells from brain, blood, bone marrow, pancreas, liver, skin, or any other organ

5   or tissue. Preferably, the donor or permeabilized cell is an interphase or mitotic somatic cell. In another preferred embodiment, the reprogramming media is modified by the enrichment or depletion of a factor, such as a DNA methyltransferase, histone deacetylase, histone, nuclear lamin, transcription factor, activator, repressor, growth factor, hormone, or cytokine. The reprogramming media may or may not contain

10 exogenous nucleotides. In other preferred embodiments, a chromatin mass in a reprogramming media or formed in a permeabilized cell is contacted with a vector having a nucleic acid encoding a gene of interest under conditions that allow homologous recombination between the nucleic acid in the vector and the corresponding nucleic acid in the genome of the chromatin mass, resulting in the alteration of the genome of the

15 chromatin mass. Due to the lack of an intact plasma membrane and the lack of a nuclear membrane, a chromatin mass in a permeabilized cell may be easier to genetically modify than a naturally-occuring cell. Preferably, the chromatin mass or nucleus is purified from the reprogramming media prior to insertion into the recipient cell or cytoplast, or the reprogrammed cell is purified prior to administration into the mammal. Preferably, the

20 donor or permeabilized cell is haploid (DNA content of n), diploid (2n), or tetraploid (4n), and the recipient cell is hypodiploid (DNA content of less than 2n), haploid, or enucleated.

Preferred donor cells, permeabilized cells, recipient cells, reprogrammed cells, and sources of cytoplasts include differentiated cells, such as epithelial cells, neural cells,

25 epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-cells, T-cells, erythrocytes, macrophages, monocytes, fibroblasts, and muscle cells; and undifferentiated cells, such as embryonic or adult stem cells. In another preferred embodiment, the donor or permeabilized cell is a differentiated cell, and the reprogrammed cell is a differentiated cell of another cell type. In yet another preferred

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embodiment, the donor or permeabilized cell is an undifferentiated cell, and the reprogrammed cell is a differentiated cell. In still another preferred embodiment, the donor or permeabilized cell is a differentiated cell, and the reprogrammed cell is an undifferentiated cell. If desired, an undifferentiated reprogrammed cell may be induced

5   to differentiate into a desired cell type *in vitro* using standard methods, such as by exposure to certain growth factors, hormones, interleukins, cytokines, or other cells. In another preferred embodiment, the undifferentiated reprogrammed cell differentiates into a desired cell type *in vivo* after administration to a mammal. In yet another preferred embodiment, the donor or permeabilized cell is a B-cell, Jurkat cell, endothelial cell,

10 epithelial cell, or fibroblast, and the reprogrammed cell is a T-cell. It is also contemplated that the nucleus or chromatin mass may be inserted into a recipient cell or cytoplast of the desired cell type or of the same cell type as the donor or permeabilized cell. In still another preferred embodiment, the donor cell, permeabilized cell, recipient cell, or recipient cytoplast is from a human or non-human mammal. In yet another

15 preferred embodiment, the donor nucleus or chromatin mass is from a transgenic cell or mammal or contains a mutation not found in the donor cell or not found in a naturally-occurring cell. The donor or permeabilized cell can be non-immortilized or naturally, spontaneously, or genetically immortilized. The donor cell, permeabilized cell, recipient cell, or cytoplast can be from a source of any age, such as an embryo, fetus, youth, or

20 adult mammal. Cells from younger sources may have acquired fewer spontaneous mutations and may have a longer life-span *in vitro* or after transplantation *in vivo*.

Preferably, a disease-causing mutation in a regulatory region, promoter, untranslated region, or coding region of a gene in a donor nucleus or chromatin mass is modified to replace the mutant sequence with a sequence that is not associated with the

25 disease. Alternatively, a nucleic acid is inserted into the donor nucleus or chromatin mass that includes a promoter operably-linked to a sequence of the gene that does not contain a mutation associated with a disease. Preferably, the sequence of the gene is substantially identical to that of a naturally-occurring gene that does not contain a polymorphism or mutation associated with a disease. Examples of mutations that may be

rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Deyfuss muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gln6 is mutated to a stop codon.

Preferred transgenic donor nuclei, chromosomes, or chromatin masses encode a heterologous MHC Class I protein having an amino acid sequence substantially identical to the sequence of an MHC Class I protein found in the mammal to whom the reprogrammed cells will be administered for therapeutic applications. Alternatively, the donor nuclei or chromatin masses may encode a heterologous MHC Class 1 protein having an amino acid sequence substantially identical to the sequence of an MHC Class 1 protein found in another mammal of the same genus or species as the recipient mammal. Reprogrammed cells that express such MHC proteins are less likely to elicit an adverse immune response when administered to the mammal. Other preferred donor nuclei or chromatin masses are modified to express a heterologous protein that inhibits the complement pathway of the recipient mammal, such as the human complement inhibitor CD59 or the human complement regulator decay accelerating factor (h-DAF) (see, for example, Ramirez *et al.*, Transplantation 15:989-998, 2000; Costa *et al.*, Xenotransplantation 6:6-16, 1999). In yet another preferred embodiment, the donor nucleus or chromatin mass has a mutation that reduces or eliminates the expression or activity of a galactosyltransferase, such as alpha(1,3)-galactosyltransferase (Tearle *et al.*, Transplantation 61:13-19, 1996; Sandrin, Immunol. Rev. 141:169-190, 1994; Costa *et al.*, Xenotransplantation 6:6-16, 1999). This enzyme modifies cell surface molecules with a carbohydrate that elicits an adverse immune response when cells expressing this galactose alpha(1,3)-galactose epitope are administered to humans. Thus, reprogrammed cells that have a lower level of expression of this epitope may have a lower incidence of rejection by the recipient mammal.

With respect to the therapeutic methods of the invention, it is not intended that the administration of reprogrammed cells to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. Preferably, the cells are administered to the mammal from which the donor or permeabilized cell is obtained. Alternatively, the donor or permeabilized cell may be obtained from a different donor mammal of the same or a different genus or species as the recipient mammal. Examples of preferred donor mammals include humans, cows, sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, rabbits, pigs, mice, rats, guinea pigs, hamsters, dogs, cats, and primates such as monkeys. The cells may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type. Additionally, one or more immunosuppressive agents, such as cyclosporin, may be administered to inhibit rejection of the transplanted cells. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions.

As used herein, by "chromatin mass" is meant more than one chromosome not enclosed by a membrane. Preferably, the chromatin mass contains all of the chromosomes of a cell. A chromatin mass containing condensed chromosomes may be formed by exposure of a nucleus to a mitotic reprogramming media (*e.g.*, a mitotic extract), or a chromatin mass may be isolated from mitotic cells as described herein. Alternatively, a chromatin mass containing decondensed or partially condensed chromosomes may be generated by exposure of a nucleus to one of the following, as

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described herein: a mitotic reprogramming media (*e.g.*, a mitotic extract) in the presence of an anti-NuMA antibody, a detergent and salt solution, or a protein kinase solution.

A chromatin mass may be formed naturally or artificially induced. An exemplary naturally-occurring chromatin mass includes a set of metaphase chromosomes, which are

5   partially or maximally condensed chromosomes that are not surrounded by a membrane and that are found in, or isolated from, a mitotic cell. Preferably, the metaphase chromosomes are discrete chromosomes that are not physically touching each other. Exemplary artificially induced chromatin masses are formed from exposure to a reprogramming media, such as a solution containing factors that promote chromosome

10   condensation, a mitotic extract, a detergent and salt solution, or a protein kinase solution. Artificially induced chromatin masses may contain discrete chromosomes that are not physically touching each other or may contain two or more chromosomes that are in physical contact.

If desired, the level of chromosome condensation may be determined using

15   standard methods by measuring the intensity of staining with the DNA stain, DAPI. As chromosomes condense, this staining intensity increases. Thus, the staining intensity of the chromosomes may be compared to the staining intensity for decondensed chromosomes in interphase (designated 0% condensed) and maximally condensed chromosomes in mitosis (designated 100% condensed). Based on this comparison, the

20   percent of maximal condensation may be determined. Preferred condensed chromatin masses are at least 50, 60, 70, 80, 90, or 100% condensed. Preferred decondensed or partially condensed chromatin masses are less than 50, 40, 30, 20, or 10% condensed.

By "nucleus" is meant a membrane-bounded organelle containing most or all of the DNA of a cell. The DNA is packaged into chromosomes in a decondensed form.

25   Preferably, the membrane encapsulating the DNA includes one or two lipid bilayers or has nucleoporins.

By "donor cell" is meant a cell from which a nucleus or chromatin mass is derived.

By "cytoplast" is meant a membrane-enclosed cytoplasm. Preferably, the cytoplast does not contain a nucleus, chromatin mass, or chromosome. Cytoplasts may be formed using standard procedures. For example, cytoplasts may be derived from nucleated or enucleated cells. Alternatively, cytoplasts may be generated using methods that do not require an intact cell to be used as the source of the cytoplasm or as the source of the membrane. In one such method, cytoplasts are produced by the formation of a membrane in the presence of cytoplasm under conditions that allow encapsulation of the cytoplasm by the membrane.

By "permeabilization" is meant the formation of pores in the plasma membrane or the partial or complete removal of the plasma membrane.

By "reprogramming media" is meant a solution that allows the removal of a factor from a nucleus, chromatin mass, or chromosome or the addition of a factor from the solution to the nucleus, chromatin mass, or chromosome. Preferably, the addition or removal of a factor increases or decreases the level of expression of an mRNA or protein in the donor cell, chromatin mass, or nucleus or in a cell containing the reprogrammed chromatin mass or nucleus. In another embodiment, incubating a permeabilized cell, chromatin mass, or nucleus in the reprogramming media alters a phenotype of the permeabilized cell or a cell containing the reprogrammed chromatin mass or nucleus relative to the phenotype of the donor cell. In yet another embodiment, incubating a permeabilized cell, chromatin mass, or nucleus in the reprogramming media causes the permeabilized cell or a cell containing the reprogrammed chromatin mass or nucleus to gain or loss an activity relative to the donor cell.

Exemplary reprogramming medias include solutions, such as buffers, that do not contain biological molecules such as proteins or nucleic acids. Such solutions are useful for the removal of one or more factors from a nucleus, chromatin mass, or chromosome. Other preferred reprogramming medias are extracts, such as cellular extracts from cell nuclei, cell cytoplasm, or a combination thereof. Yet other reprogramming medias are solutions or extracts to which one or more naturally-occurring or recombinant factors (*e.g.,* nucleic acids or proteins such as DNA methyltransferases, histone deacetylases,

histones, nuclear lamins, transcription factors, activators, repressors, growth factors, hormones, or cytokines) have been added, or extracts from which one or more factors have been removed. Still other reprogramming medias include detergent and salt solutions and protein kinase solutions. In some embodiments, the reprogramming media contains an anti-NuMA antibody. By "interphase reprogramming media" is meant a media (*e.g.*, an interphase cell extract) that induces chromatin decondensation and nuclear envelope formation. By "mitotic reprogramming media" is meant a media (*e.g.*, a mitotic cell extract) that induces chromatin condensation and nuclear envelope breakdown. If desired, multiple reprogramming media may be used simultaneously or sequentially to reprogram a donor cell, nucleus, or chromatin mass.

By "addition of a factor" is meant the binding of a factor to chromatin, a chromosome, or a component of the nuclear envelope, such as the nuclear membrane or nuclear matrix. Alternatively, the factor is imported into the nucleus so that it is bounded or encapsulated by the nuclear envelope. Preferably, the amount of factor that is bound to a chromosome or located in the nucleus increases by at least 25, 50, 75, 100, 200, or 500%.

By "removal of factor" is meant the dissociation of a factor from chromatin, a chromosome, or a component of the nuclear envelope, such as the nuclear membrane or nuclear matrix. Alternatively, the factor is exported out of the nucleus so that it is no longer bounded or encapsulated by the nuclear envelope. Preferably, the amount of factor that is bound to a chromosome or located in the nucleus decreases by at least 25, 50, 75, 100, 200, or 500%.

By "enrichment or depletion of a factor" is meant the addition or removal of a naturally-occurring or recombinant factor by at least 20, 40, 60, 80, or 100% of the amount of the factor originally present in the reprogramming media. Alternatively, a naturally-occurring or recombinant factor that is not naturally present in the reprogramming media may be added. Preferred factors include proteins such as DNA methyltransferases, histone deacetylases, histones, nuclear lamins, transcription factors, activators, repressors, growth factors, cytokines, and hormones; membrane vesicles; and

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organelles. In one preferred embodiment, the factor is purified prior to being added to the reprogramming media, as described below. Alternatively, one of the purification methods described below may be used to remove an undesired factor from the reprogramming media.

5   By "purified" is meant separated from other components that naturally accompany it. Typically, a factor is substantially pure when it is at least 50%, by weight, free from proteins, antibodies, and naturally-occurring organic molecules with which it is naturally associated. Preferably, the factor is at least 75%, more preferably, at least 90%, and most preferably, at least 99%, by weight, pure. A substantially pure factor may be obtained by

10   chemical synthesis, separation of the factor from natural sources, or production of the factor in a recombinant host cell that does not naturally produce the factor. Proteins, vesicles, chromosomes, nuclei, and other organelles may be purified by one skilled in the art using standard techniques such as those described by Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000). The factor is preferably at

15   least 2, 5, or 10 times as pure as the starting material, as measured using polyacrylamide gel electrophoresis, column chromatography, optical density, HPLC analysis, or western analysis (Ausubel et al., supra). Preferred methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, and panning with a plate-bound antibody.

20   By "mRNA or protein specific for one cell type" is meant an mRNA or protein that is expressed in one cell type at a level that is at least 10, 20, 50, 75, or 100 fold greater than the expression level in all other cell types. Preferably, the mRNA or protein is only expressed in one cell type.

       By "mutation" is meant an alteration in a naturally-occurring or reference nucleic

25   acid sequence, such as an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, or missense mutation. Preferably, the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid alteration from a naturally-occurring sequence. Examples of recombinant DNA techniques for altering the genomic sequence of a cell, embryo, fetus, or mammal include inserting a DNA sequence from

another organism (*e.g.*, a human) into the genome, deleting one or more DNA sequences, and introducing one or more base mutations (*e.g.,* site-directed or random mutations) into a target DNA sequence. Examples of methods for producing these modifications include retroviral insertion, artificial chromosome techniques, gene insertion, random insertion

5   with tissue specific promoters, homologous recombination, gene targeting, transposable elements, and any other method for introducing foreign DNA. All of these techniques are well known to those skilled in the art of molecular biology (see, for example, Ausubel *et al., supra*). Chromatin masses, chromosomes, and nuclei from transgenic cells, tissues, organs, or mammals containing modified DNA may be used in the methods of the

10   invention.

By "substantially identical" is meant having a sequence that is at least 60, 70, 80, 90, or 100% identical to that of another sequence or to a naturally-occurring sequence. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics

15   Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

By "immortilized" is meant capable of undergoing at least 25, 50, 75, 90, or 95%

20   more cell divisions than a naturally-occurring control cell of the same cell type, genus, and species as the immortalized cell or than the donor cell from which the immortalized cell was derived. Preferably, an immortalized cell is capable of undergoing at least 2, 5, 10, or 20-fold more cell divisions than the control cell. More preferably, the immortalized cell is capable of undergoing an unlimited number of cell divisions.

25   Examples of immortalized cells include cells that naturally acquire a mutation *in vivo* or *in vitro* that alters their normal growth-regulating process. Other preferred immortalized cells include hybridoma cells which are generated using standard techniques for fusion of a myeloma with a B-cell (Mocikat, J. Immunol. Methods 225:185-189, 1999; Jonak *et al.*, Hum. Antibodies Hybridomas 3:177-185, 1992; Srikumaran *et al.*, Science 220:522,

1983). Still other preferred immortalized cells include cells that have been genetically modified to express an oncogene, such as ras, myc, abl, bcl2, or neu, or that have been infected with a transforming DNA or RNA virus, such as Epstein Barr virus or SV40 virus (Kumar *et al.*, Immunol. Lett. 65:153-159, 1999; Knight *et al.*, Proc. Nat. Acad. Sci.

5 USA 85:3130-3134, 1988; Shammah *et al.*, J. Immunol. Methods 160-19-25, 1993; Gustafsson and Hinkula, Hum. Antibodies Hybridomas 5:98-104, 1994; Kataoka *et al.*, Differentiation 62:201-211, 1997; Chatelut *et al.*, Scand. J. Immunol. 48:659-666, 1998). Cells can also be genetically modified to express the telomerase gene (Roques *et al.*, Cancer Res. 61:8405-8507, 2001).

10 By "non-immortilized" is meant not immortalized as described above.

The present invention provides a number of advantages related to the alteration of cell fate. For example, these methods may be generally applied to produce cells of any desired cell type. Because these methods involve incubating a nucleus, a chromatin mass, or a permeabilized cell in a reprogramming media (*e.g.*, a cell extract) to allow

15 reprogramming, the efficiency of reprogramming may be enhanced by adding factors to the reprogramming media that facilitate reprogramming or by removing factors that inhibit reprogramming. These reprogrammed cells may be transplanted into mammals for the treatment or prevention of conditions involving damage or deficiency of a particular cell type. If desired, the reprogrammed cells may be manipulated using

20 standard molecular biology techniques to correct a disease-causing mutation before administering the cells to a recipient mammal.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

25 <div align="center">Brief Description of the Drawings</div>

The application file contains drawings executed in color (Figs. 13, 14A, 14B, 14C, 15, and 17A). Copies of this patent or patent application with color drawings will be provided by the Office upon request and payment of the necessary fee.

Figure 1 is an illustration of a gel showing the amount of IL-2 mRNA synthesized by human T-cells stimulated with anti-CD3 antibodies for the indicated lengths of time. Lanes "30c," "60c," and "120c" refer to mock-stimulated T-cells.

Figure 2 is an illustration of nuclei purified from resting T-cells.

Figure 3A is the immunofluorescence analysis of nuclear uptake and binding of the T-cell specific transcription factor NFAT in cell-free reprogramming extracts. For this assay, nuclei purified from resting T-cells ("Input" nuclei) were incubated with reprogramming extract from stimulated T-cells for 30 minutes. Figure 3B in an illustration of an immunoblot showing the nuclear uptake of NFAT, c-Jun/AP1, NFκB, Oct1, and MAP kinase (Erk1 and Erk2). Input nuclei ("Input") and nuclei incubated in either a stimulated extract ("SE"), a control extract prepared from unstimulated T-cells (denoted "USE" for unstimulated extract), or a stimulated extract containing a monoclonal antibody against nucleoporins
("SE + mAb414") were analyzed. Figure 3C is a bar graph showing the percentage of transcription factors bound to DNA, reported as the mean ± the standard deviation.

Figure 4A is an illustration of an immunoblot showing the DNA-binding of the chromatin remodeling SWI/SNF complex in T-cell nuclei exposed to a buffer control, an unstimulated extract, or a stimulated extract for 30 minutes and sedimented through sucrose. Figure 4B is a graph showing the percentage of DNA bound and unbound SWI/SNF complex in nuclei incubated in extract for the indicated lengths of time, as assessed using a nuclear retention assay. The SWI/SNF complex was visualized on Western blots using anti-BRG1 antibodies. Figure 4C is an illustration of an immunoblot showing the nuclear retention of the SWI/SNF complex in stimulated T-cells *in vivo*. For this assay, T-cells were stimulated with anti-CD3 antibodies for 30 minutes, and the soluble and DNA bound fractions of the SWI/SNF complex were assessed as described for Fig. 4A. Figure 4D is an illustration of an immunoblot showing hyperacetylation of the IL-2 gene of T-cell nuclei *in vivo* and *in vitro*. An MNAse-soluble chromatin fraction was prepared from resting and anti-CD3-stimulated T-cells. The immunoblot shows the binding of an IL-2 probe and a control β-actin probe to DNA from the immunoprecipitate

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of the chromatin fraction with an anti-acetylated histone H4 (H4ac) probe ("bound") and the binding to DNA in the supernatant ("unbound") fractions.

Figure 5A is a gel showing the synthesis of IL-2 mRNA in cell-free extracts. Resting T-cell nuclei ("Input nuclei") were incubated for 30 minutes at 30°C in an unstimulated extract or a stimulated extract. As controls, nuclei were incubated in a stimulated extract containing either 100 μg/ml RNAse A, 100 μg/ml DNAse I, mAb414 antibodies, or the lectin WGA. Figure 5B is a gel showing that IL-2 mRNA production *in vitro* is dependent on PolII transcription. Nuclei were exposed for 30 minutes to a stimulated extract containing increasing concentrations of the RNA PolII inhibitor actinomycin D (0, 5, 10, 50, 100 and 500 nM), and IL-2 mRNA synthesis was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Figure 5C is an illustration of a gel showing that IL-2 mRNA production was induced in nuclei exposed to a T-cell extract treated with anti-CD3 antibodies but not induced by exposure to extracts treated with anti-CD3 antibodies from the B-cell line Reh, human 293T fibroblasts, or HeLa cells. The arrows in the illustration point to the 467 base pair IL-2 RT-PCR product.

Figure 6A is the immunofluorescence analysis showing nuclear uptake and binding of the T-cell specific transcription factor NFAT by nuclei from the B-cell line Reh, 293T fibroblasts, or HeLa cells that have been incubated with the stimulated T-cell extract, as described for Fig. 3A. Figure 6B in an illustration of an immunoblot showing the nuclear uptake of NFAT, c-Jun/AP1, and NFκB by these nuclei, as described for Fig. 3B.

Figure 7 is a picture of a gel showing RT-PCR analysis of IL-2 mRNA synthesis in human peripheral blood T-cells stimulated with anti-CD3 antibodies. At indicated time points, cells were lysed, and total RNA was isolated for RT-PCR analysis using *IL-2*-specific primers. Mock (H_2O)-stimulated cells were analyzed at 30, 60, and 120 minute time points ("30c," "60c," "120c")

Figures 8A-8D are pictures and graphs that illustrate nuclear uptake and chromatin binding of transcriptional activators of the *IL-2* gene in stimulated T-cell extract. Fig. 8A

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is a picture of nuclei purified from quiescent T-cells ("Input nuclei") and incubated in stimulated extract for 30 minutes. Nuclear integrity was assessed by phase contrast microscopy and membrane labeling with 10 μg/ml of the lipophilic dye $DiOC_6$ (bar, 10 μm). Fig. 8B is a picture of an immunoblot showing the relative levels of NFAT, AP-1,

5   NFκB, Oct-1, Erk,and an exogenous BSA-NLS conjugate in input resting T-cell nuclei ("Input nuclei"), input unstimulated extract, and input stimulated extract. Nuclear uptake of these factors was examined in nuclei exposed to stimulated extract, unstimulated extract, or stimulated extract containing the nuclear pore blocking antibody mAb414. Blots were also probed using anti-histone H4 antibodies as a gel loading control. Fig. 8C

10   is a graph of the immunoblotting analysis of intranuclear anchoring of imported transcription factors in nuclei exposed to unstimulated extract or stimulated extract. Intranuclear anchoring was assessed using a nuclear retention assay and immunoblotting analysis of Triton X-100 insoluble ("bound") fractions. The percentage (mean±SD) of bound factors was determined by densitometric analysis of duplicate blots. Fig. 8D is a

15   picture of immunoblotting analysis of nuclear matrix ("Mtx") and chromatin ("Chr") fractions prepared from nuclei exposed to stimulated extract. NuMA and RNA Pol IIo were used as markers of the nuclear matrix and chromatin, respectively. Anti-NuMA antibodies and anti- Pol IIo mAb CC3 were gifts from D. Compton (Dartmouth Medical School, Hanover, NH, USA) and M. Vincent (Université Laval, Quebec, Canada),

20   respectively.

        Figures 9A-9E illustrate chromatin remodeling in resting T-cell nuclei exposed to a stimulated T-cell extract. Fig. 9A is a gel showing the anchoring of the chromatin remodeling complex SWI/SNF in nuclei exposed to stimulated extract. Purified quiescent T-cell nuclei ("Input nuclei") were incubated in cell lysis buffer ("Buffer"),

25   unstimulated extract, or stimulated extract for 30 minutes, and sedimented through sucrose. Free and bound SWI/SNF fractions were assessed using the nuclear retention assay and immunoblotting with anti-BRG1 antibodies. Fig. 9B is a graph of the percentage of bound SWI/SNF, as determined using the nuclear retention assay during

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incubation of nuclei in stimulated extract or unstimulated extract. Fig. 9C is a gel showing nuclear retention of SWI/SNF in cultured stimulated T-cells. T-cells were stimulated with anti-CD3 antibodies and after 30 minutes, intranuclear bound and free fractions of SWI/SNF were assessed as in Fig. 9A. Fig. 9D is a graph of the ATPase

5   activity of the SWI/SNF complex. After incubation of resting T-cell nuclei in stimulated extract or unstimulated extract for 30 minutes, SWI/SNF was immunoprecipitated from nuclear lysates using anti-BRG1 antibodies and hydrolysis of 1 nM exogenous ATP ("ATP +") by the immune precipitate ("BRG1-IP") was determined in a luminometric assay. Control precipitations were carried out using pre-immune IgGs ("Pre-I IgG").

10   Top rows show an anti-BRG1 immunoblot of the BRG1-IPs. Where indicated, BRG1-IP was treated with 100 μg/ml DNAse I for 15 minutes prior to the assay ("DNAse +"). Relative ATPase activities are expressed as mean (±SD) relative light units (RLU) in the assay subtracted to the RLU of the control pre-immune precipitate (RLU=2,700, set to zero). Fig. 9E is a gel showing the hyperacetylation of the *IL-2* promoter in T-cell nuclei

15   in culture and *in vitro*. Microccocal nuclease-soluble chromatin ("Input") was prepared from unstimulated ("Unstim.") and anti-CD3-stimulated ("Stim.") T-cells. AcH4 was immunoprecipitated, and DNA isolated from anti-acH4 precipitates ("Bound") and supernatant ("Unbound") fractions. DNA was dot-blotted and hybridized with an *IL-2* promoter-specific probe and a control *β-actin* probe.

20   Figures 10A-10D are gels showing the transcription of IL-2 mRNA by T-cell and non-T-cell nuclei in cell-free extracts. Fig. 10A is a gel showing transcription by resting T-cell nuclei ("Input nuclei") incubated in stimulated extract ("Nuclei-SE") or unstimulated extract ("Nuclei-USE"). As controls, nuclei were incubated in stimulated extract containing either 100 μg/ml RNAse A, 100 μg/ml DNAse I, mAb414, or wheat

25   germ agglutinin ("WGA"). At the end of the incubation, total RNA was isolated from the reaction mix, and 15 ng was used as the template for RT-PCR using *IL-2*-specific primers. Input stimulated extract and stimulated extract containing 1.2 μg total RNA isolated from IL-2 producing T-cells ("Pos. control SE") were analyzed as controls. Fig.

10B is a picture of a gel showing that IL-2 mRNA synthesis *in vitro* is RNA Pol II-dependent. Resting T-cell nuclei were exposed to stimulated extract containing 0, 5, 10, 50, 100 or 500 nM of the RNA Pol II inhibitor, actinomycin D. IL-2 mRNA synthesis was analyzed by RT-PCR at the end of incubation. Fig. 10C is a gel showing

5   transcription by nuclei from primary HUVEC cells, NT2 cells, and Hela cells that were reprogrammed for 2 hours in stimulated extract or unstimulated extract. As controls, nuclei were incubated in stimulated extract containing either 100 μg/ml RNAse A, mAb414, or 50 nM actinomycin D ("ActD"). Total RNA was isolated, and IL-2 RNA synthesis was examined by RT-PCR. Fig. 10D is a picture of a gel showing transcription

10   by resting T-cell nuclei that were incubated for two hours in stimulated extract, or in extracts from 293T, HeLa, or Bjab cells, all prepared after treating each cell type with anti-CD3 and cross-linking antibodies to mimic T-cell stimulation. IL-2 mRNA synthesis in each extract was analyzed by RT-PCR.

         Figures 11A-11C demonstrate the nuclear import and chromatin binding of

15   transcriptional activators of the *IL-2* gene in 293T fibroblast nuclei exposed to stimulated T-cell extract. Fig. 11A is a picture of nuclei purified from 293T fibroblasts (0 min) and incubated in stimulated T-cell extract. Uptake of NFAT was examined by immunofluorescence (bar, 10 μm). Fig. 11B is a picture of immunoblotting analysis showing nuclear uptake of NFAT, AP-1, NFκB, Oct-1, and BSA-NLS in input 293T

20   nuclei and 293T nuclei exposed to either stimulated T-cell extract, unstimulated T-cell extract, or stimulated T-cell extract and mAb414. Anti-histone H4 antibodies were used as a loading control. Fig. 11C is a picture of an immunoblot showing nuclear matrix ("Mtx") and chromatin ("Chr") fractions prepared from 293T nuclei treated with stimulated T-cell extract.

25   Figures 12A-2E demonstrate chromatin remodeling and activation of the *IL-2* gene in stimulated T-cell extract. Fig. 12A is a gel showing intranucleuear anchoring of the human SWI/SNF complex. In the left section of the immunoblot, nuclei were isolated from resting ("-") or anti-CD3-stimulated ("α-CD3") T-cells, and intranuclear free and

bound SWI/SNF assessed by immunoblotting of detergent-soluble and insoluble nuclear fractions using anti-BRG1 antibodies. In the right section of the immunoblot, free and bound SWI/SNF fractions were visualized in 293T nuclei incubated in stimulated T-cell extract and sedimented through sucrose. Fig. 12B is a graph of the percent of bound

5   SWI/SNF in 293T nuclei exposed to stimulated T-cell extract or unstimulated T-cell extract, based on densitometric analysis of duplicate blots. Fig. 12C is a graph of the ATPase activity of the SWI/SNF complex. Following exposure of 293T nuclei to stimulated T-cell extract or unstimulated T-cell extract, SWI/SNF was immunoprecipitated from nuclear lysates using anti-BRG1 antibodies and hydrolysis of 1

10   nM exogenous ATP ("ATP +") by the immune precipitate ("BRG1-IP") was determined in a luminometric assay. Control precipitations were carried out using pre-immune IgGs ("Pre-I IgG"). ATP levels are expressed as mean (±SD) relative light units (RLU). Elevated ATP levels reflect low ATPase activity of the BRG1-IP. Fig. 12D is an immunoblot showing the hyperacetylation of the *IL-2* locus in 293T nuclei. MNase-

15   soluble chromatin was prepared from input 293T nuclei ("Input N"), and the nuclei were exposed to unstimulated T-cell extract or stimulated T-cell extract. Acetylated H4 was immunoprecipitated, and DNA was isolated from anti-acH4 precipitate ("Bound") and supernatant ("Unbound") fractions. DNA was dot-blotted and hybridized to an *IL-2* probe (top rows) and a control *β-actin* probe (bottom rows). Fig. 12 is a picture of a set

20   of gels showing transcription of the *IL-2* gene. Nuclei from 293T, NT2, and resting T-cells were incubated for two hours in unstimulated T-cell extract ("Nuclei/UTE") or stimulated T-cell extract ("Nuclei/STE"). As controls, nuclei were incubated in stimulated T-cell extract containing either 100 µg/ml RNAse A, mAb414, or 50 nM actinomycin D ("ActD"). RNA was isolated from the reaction mix, and *IL-2*

25   transcription was examined by RT-PCR.

Figure 13 is a graph demonstrating that 293T fibroblasts reprogrammed in the Jurkat-TAg extract display altered gene expression. Relative mRNA levels in 293T-cells incubated in stimulated Jurkat-TAg extract or in control 293T extract were compared using a cytokine gene expression array. Bars represent fold increase or decrease in

transcription of indicated genes in Jurkat extract-treated cells, measured as the ratio of reprogrammed/control probe hybridization signal strength. Genes with an over two-fold increase or decrease in expression level are shown. Different color backgrounds separate distinct gene groups.

5   Figures 14A-14C demonstrate that 293T fibroblasts reprogrammed in Jurkat-TAg extract exhibit hematopoietic cell markers and function. Fig. 14A is a picture of 293T-cells exposed to a control 293T extract (top row) or a stimulated Jurkat-TAg extract (middle row), and Jurkat-TAg cells (bottom row) and analyzed by immunofluorescence using indicated FITC-conjugated antibodies. CD3, CD4, CD8 were detected at four days

10   post-reprogramming; CD45 was detected at 11 days post-reprogramming. The Cγ subunit of PKA ("PKA-Cγ") was examined as a positive control. Fig. 14B is a picture of the immunofluorescence analysis of the cells using anti-TCRαβ antibodies at 11 days post-reprogramming. DNA was labeled with propidium iodide. Fig. 14C is a picture of each cell type stimulated with anti-CD3 antibodies and PMA for 24 hours, starting at two

15   days post-reprogramming. Stimulated and unstimulated cells were analyzed by immunofluorescence using anti-IL-2Rβ (green) and anti-IL-2Rα (red) antibodies. Stimulation of Jurkat cells and Jurkat extract-treated 293T fibroblasts elicited IL-2-Rα synthesis. DNA (blue) was labeled with Hoechst 33342. Merged images are shown (bars, 10 μm).

20   Figure 15 is a picture of the immunofluorescence analysis of 293T fibroblasts reprogrammed in NT2 extract, demonstrating that the reprogrammed cells express the neurofilament protein NF-200. NT2 or control 293T extract-treated fibroblasts were examined by immunofluorescence using anti-NF200 antibodies at 15 days post-reprogramming. DNA was labeled with Hoechst 33342 (bar, 10 μm).

25   Figure 16A is a set of pictures showing the morphology of NIH3T3 fibroblasts and mouse ES cells. Fig. 16B is a set of pictures showing the morphology of NIH3T3 fibroblasts reprogrammed in mouse embryonic stem cell extract, fibroblasts mock-

reprogrammed in NIH3T3 extract, and intact NIH3T3 cells exposed to ES cell extract. Phase contrast micrographs are shown (bars, 20 μm).

Figures 17A and 17B are pictures of the immunofluorescence and immunoblotting analysis, respectively, of Oct4 in NIH3T3 cells, embryonic stem cells and NIH3T3

5   fibroblasts reprogrammed in embryonic stem cell extract ("NIH/ES ext."; day-4 post-reprogramming). NIH3T3 cells exposed to a control NIH3T3 cell extract do not express Oct4 ("NIH/NIH ext.").

Figure 18 is a picture of a membrane showing the detection of alkaline phosphatase activity in mouse embryonic stem cells. The top row contains lysates of

10   NIH3T3 cells, and the bottom row contains lysates of embryonic stem cells. Two and 6 μl lysate were applied onto the membrane (protein concentration is ~20 μg/μl).

Figure 19 is a picture of a membrane showing the detection of alkaline phosphatase activity in NIH3T3 cells reprogrammed in the embryonic stem cell extract.

15   <u>Detailed Description</u>

We have developed novel methods of reprogramming cells by exposing them or their genetic material to a reprogramming media (*e.g.*, a cell extract). This reprogramming refers to decreasing or eliminating the expression of genes specific for the donor cell or increasing the expression of genes specific for another cell type. For

20   example, we have shown that incubation of nuclei from resting T-cells, B-cells, or fibroblasts in an extract from stimulated T-cells results in migration of a T-cell specific transcription factor from the extract into the nuclei. Additionally, the reprogramming of nuclei from resting T-cells, fibroblasts, endothelial (HUVEC), differentiated epithelial (HeLa), and neuronal precursor (NT2) cells induced expression of the IL-2 gene, a gene

25   that is otherwise repressed by the nuclei. Reprogramming of resting T-cell and fibroblast nuclei also induced hyperacetylation of the IL-2 gene and intranuclear anchoring of a chromatin remodeling complex. Thus, reprogramming media such as extracts may be used to alter the expression profile of the genetic material of a donor cell such that it resembles that of the cells used to prepare the reprogramming media.

The methods for reprogramming a cell that are described herein may be used to convert a cell into another cell-type that is closely related by origin or character. For example, members of the connective-tissue family, such as fibroblasts, smooth muscle cells, osteoblasts, adiopocytes, and chrondrocytes, may be interconverted using these

5   methods. Additionally, hepatocytes may be converted into insulin-producing B-cells because both of these cell types express many of the same genes. Alternatively, a cell may be converted into a desired cell type that is distantly related to the donor cell and thus shares few or no characteristics or functions with the donor cell.

In one such reprogramming method, a nucleus from an interphase donor cell is

10   incubated in a reprogramming media prepared from interphase cells (*e.g.*, an interphase cell extract) under conditions that allow export of factors, such as transcription regulatory factors, from the nucleus and the import of factors from the reprogramming media into the nucleus. The nucleus is then inserted into a recipient cell or cytoplast, forming a reprogrammed cell. Preferably, the cells used to prepare the interphase reprogramming

15   media are the cell type one wishes the reprogrammed cell to become. Due to the different factors in the nucleus of the reprogrammed cell compared to that of the donor cell, the reprogrammed cell expresses a different set of mRNA and protein molecules and thus has a different phenotype than that of the donor cell. To achieve optimum reprogramming efficiency, 2, 3, 5, or more rounds of reprogramming may be performed.

20   The reprogrammed cells may also be cultured under conditions promoting sustained changes in cell function. For example, the cells may be cultured with additional components such as antigens, interleukins, growth factors, cytokines, or other cells. The reprogrammed cells can also be transplanted into a host animal or patient, in the organ where they are supposed to function. Local environment cues may facilitate

25   reprogramming.

In a related method, the nucleus from a donor interphase cell is incubated in a mitotic reprogramming media (*e.g.*, a mitotic cell extract), a detergent and salt solution, or a protein kinase solution to promote nuclear envelope dissolution and possibly chromatin condensation, forming a chromatin mass. This nuclear envelope breakdown

and chromatin condensation facilitate the release of factors from the chromatin mass. Alternatively, a chromatin mass may be isolated from a donor mitotic cell. In one embodiment of this method, the chromatin mass is inserted into a recipient cell or cytoplast of the desired cell type. After this nuclear transfer, a nucleus is reformed from the donor chromatin mass. Additionally, desired factors from the cytoplasm of the recipient cell or cytoplast migrate into the nucleus and bind the exogenous chromosomes, resulting in the expression of desired genes by the reprogrammed cell. To promote the sustained expression of the desired genes, 2, 3, 5, or more rounds of reprogramming may be performed, and the reprogrammed cells may also be cultured with additional components such as antigens, interleukins, growth factors, cytokines, or other cells.

In another embodiment of this method, the chromatin mass is first incubated in an interphase reprogramming media (e.g., an interphase cell extract) as described above to further promote the release of undesirable factors from the chromatin mass and the binding of desirable factors from the interphase reprogramming media to the chromatin mass. The incubation in the interphase reprogramming media also results in the formation of a nuclear membrane, encapsulating the chromatin mass and desired factors from the reprogramming media. The reformed nucleus is then inserted into a recipient cell or cytoplast of the desired cell type or of any other cell type.

As an alternative to isolating nuclei or chromatin masses from donor cells for subsequent incubation in a reprogramming media, donor cells may be gently permeabilized and incubated in the reprogramming media (e.g., a cell extract). Permeabilization of the plasma membrane allows factors to enter and leave the cell. The cells may either be incubated in an interphase reprogramming media to allow the nucleus to remain membrane-bounded or with a mitotic reprogramming media to allow the dissolution of the nuclear membrane and formation of a chromatin mass. After incubation in the reprogramming media, the plasma membrane may be resealed, trapping desired factors from the reprogramming media inside the cell. If desired, this reprogramming method can be repeated 1, 2, 3, 4, 5, or more times. For example, after the resealed cell is cultured for a certain length of time (e.g., after 2 days, 7 days, 14 days,

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3 weeks, 4 weeks, 8 weeks, or longer) in the presence or absence of factors such as antigens, interleukins, growth factors, cytokines, or other cells to promote reprogramming, the cells are permeabilized and subjected to an additional round of reprogramming. Additional cycles of reprogramming may result in more stable and

5   heritable epigenetic changes and in prolonged expression of the phenotype or proteins of interest from the reprogrammed cells.

This whole cell reprogramming method was utilized to reprogram permeabilized, human fibroblast cells using an extract from the lymphoblastic leukemia cell line, Jurkat-TAg (hereafter referred to as Jurkat), resulting in the remodeling of chromatin, activation

10   of lymphoid-specific genes, and establishment of a T-cell-specific activity. For example, T-cell-specific antigens such as the CD3-T-cell receptor (TCR) complex were expressed by the reprogrammed cells, and the IL-2 receptor was assembled in response to CD3-TCR stimulation of these cells. After exposure to an NT2 neuronal precursor cell extract, permeabilized fibroblasts expressed a neurofilament protein and extended neurite-like

15   outgrowths in culture. Fibroblasts were also reprogrammed into cells resembling embryonic stem cells.

Reprogrammed cells generated from these methods may be used to replace cells in a mammal in need of a particular cell type. The reprogramming methods may be used to either directly produce cells of the desired cell type or to produce undifferentiated cells

20   which may be subsequently differentiated into the desired cell type. For example, stem cells may be differentiated *in vitro* by culturing them under the appropriate conditions or differentiated *in vivo* after administration to an appropriate region in a mammal. To optimize phenotypic and functional changes, reprogrammed cells can be transplanted into the organ (*e.g.*, a heart) where they are intended to function in an animal model or in

25   human patients shortly after reprogramming (*e.g.*, after 1, 2, 3, 5, 7, or more days). Reprogrammed cells implanted in an organ may be reprogrammed to a greater extent than cells grown in culture prior to transplantation. Cells implanted in an animal organ may be removed from the organ and transplanted into a recipient mammal such as a human, or the animal organ may be transplanted into the recipient.

To increase the length of time the cell, nuclei, or chromatin mass may be reprogrammed *in vitro* prior to administration to a mammal for the treatment of disease, the donor cell may be optionally modified by the transient transfection of a plasmid containing an oncogene flanked by loxP sites for the Cre recombinase and containing a nucleic acid encoding the Cre recombinase under the control of an inducible promoter (Cheng *et al.*, Nucleic Acids Res. 28(24):E108, 2000). The insertion of this plasmid results in the controlled immortalization of the cell. After the cell is reprogrammed into the desired cell-type and is ready to be administered to a mammal, the loxP-oncogene-loxP cassette may be removed from the plasmid by the induction of the Cre recombinase which causes site-specific recombination and loss of the cassette from the plasmid. Due to the removal of the cassette containing the oncogene, the cell is no longer immortalized and may be administered to the mammal without causing the formation of a cancerous tumor.

Examples of medical applications for these reprogrammed cells include the administration of neuronal cells to an appropriate area in the human nervous system to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS; or a spinal cord injury. In particular, degenerating or injured neuronal cells may be replaced by the corresponding cells from a mammal. This transplantation method may also be used to treat, prevent, or stabilize autoimmune diseases including, but not limited to, insulin dependent diabetes mellitus, rheumatoid arthritis, pemphigus vulgaris, multiple sclerosis, and myasthenia gravis. In these procedures, the cells that are attacked by the recipient's own immune system may be replaced by transplanted cells. In particular, insulin-producing cells may be administered to the mammal for the treatment or prevention of diabetes, or oligodendroglial precursor cells may be transplanted for the treatment or prevention of multiple sclerosis. For the treatment or prevention of endocrine conditions, reprogrammed cells that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be

administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that reprogrammed cells may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver,

5   lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, or uterus.

Reprogrammed cells may also be combined with a matrix to form a tissue or organ *in vitro* or *in vivo* that may be used to repair or replace a tissue or organ in a recipient mammal. For example, reprogrammed cells may be cultured *in vitro* in the presence of a

10   matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed *in vitro* by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are

15   transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, reprogrammed cells such as chondrocytes or osteocytes are cultured *in vitro* in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the

20   cells and a matrix may be administered to a mammal for the formation of the desired tissue *in vivo*. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluronic acid-based polymers,

25   and synthetic polymer matrices containing polyanhydride, polyorthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Patent Numbers 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

These methods are described further below. It is noted that any of the methods described below can be performed with reprogramming media other than cell extracts.

For example, a reprogramming media can be formed by adding one or more naturally-occurring or recombinant factors (*e.g.*, nucleic acids or proteins such as DNA methyltransferases, histone deacetylases, histones, nuclear lamins, transcription factors, activators, repressors, growth factors, hormones, or cytokines) to a solution, such as a

5   buffer. Preferably, one or more of the factors are specific for the cell type one wishes the donor cell to become.

Example 1: One-step *in vitro* reprogramming method

In the following method for reprogramming cells, nuclei are isolated from

10   interphase cells and incubated in an interphase reprogramming media (*e.g.*, an interphase cell extract) under conditions that allow the addition of factors from the reprogramming media to the nuclei or the removal of factors from the nuclei. Preferably, the nuclei remain membrane-bounded during this incubation. The reprogrammed nuclei are then isolated from the reprogramming media and inserted into recipient cells or cytoplasts.

15

Isolation of nuclei

Preferably, cells from the subject who will receive the reprogrammed cells are used as the source of donor nuclei. However, cells from other members of the same species or members of a different species or genus may be used. As many as several million nuclei

20   may be isolated from synchronized or unsynchronized cell populations in culture. The cell populations may be synchronized naturally or chemically. Preferably, at least 40, 60, 80, 90, or 100% of the cells in a population are arrested in interphase, such as in one or more of the following phases of the cell cycle: G_o, G_1, S, or G_2, using standard procedures.

25   To accomplish this, cells may be incubated, for example, in low serum, such as 5%, 2%, or 0% serum, for 1, 2, 3, or more days to increase the percentage of cells in G_o phase. To synchronize cells in G_1, the cells may be grown to confluence as attached cells and then incubated in 0.5-1 µg/ml nocodazole (Sigma Chemicals, St. Louis, MO) for 17-20 hours, as described previously (see, for example, Collas *et al.*, 1999 and references

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therein). The flasks containing the attached cells are shaken vigorously by repeatedly tapping the flasks with one hand, resulting in the detachment of mitotic cells and G_1 phase doublets. The G_1 phase doublets are pairs of elongated cells at the end of the division process that are still connected by a thin bridge. Detached G_1 phase doublets

5   may be isolated from the media based on this characteristic doublet structure. The G_1 phase doublets may remain attached or may divide into two separate cells after isolation.

To increase the percentage of cells in S phase, the cells may be cultured in the presence of aphidicolin which inhibits DNA polymerase-α and thus inhibits DNA synthesis and arrests cells in S phase. Alternatively, cells may be incubated in the

10   presence of excess thymidine. The resulting high intracellular concentration of thymidine relative to that of other nucleotides also inhibits DNA polymerase.

Cells may be synchronized in G_2 by incubating the cells in the presence of aphidicolin to arrest them in S phase and then washing the cells three times by repeated centrifugation and resuspension in phosphate buffered saline (PBS), as described herein.

15   The cells are then incubated for a length of time sufficient for cells to enter G_2 phase. For example, cells with a doubling time of approximately 24 hours, may be incubated for between 6 and 12 hours to allow them to enter G_2 phase. For cells with shorter or longer doubling times, the incubation time may be adjusted accordingly.

The synchronized or unsynchronized cells are harvested in PBS using standard

20   procedures, and several washing steps are performed to transfer the cells from their original media into a hypotonic buffer (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF). For example, the cells may be washed with 50 ml of PBS and pelleted by centrifugation at 500 x g for 10 minutes at 4°C. The PBS

25   supernatant is decanted, and the pelleted cells are resuspended in 50 ml of PBS and centrifuged, as described above. After this centrifugation, the pelleted cells are resuspended in 20-50 volumes of ice-cold hypotonic buffer and centrifuged at 500 x g for 10 minutes at 4°C. The supernatant is again discarded and approximately 20 volumes of hypotonic buffer are added to the cell pellet. The cells are carefully resuspended in this

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buffer and incubated on ice for at least one hour, resulting in the gradual swelling of the cells.

To allow isolation of the nuclei from the cells, the cells are lysed using standard procedures. For example, 2-5 ml of the cell suspension may be transferred to a glass homogenizer and Dounce homogenized using an initial 10-20 strokes of a tight-fitting pestle. Alternatively, the cell suspension is homogenized using a motorized mixer (e.g., Ultraturrax). If desired, cell lysis may be monitored using phase contrast microscopy at 40-fold magnification. During this homogenization, the nuclei should remain intact and most or preferably all of the originally attached cytoplasmic components such as vesicles, organelles, and proteins should be released from the nuclei. If necessary, 1-20 μg/ml of the cytoskeletal inhibitors, cytochalasin B or cytochalasin D, may be added to the aforementioned hypotonic buffer to facilitate this process. Homogenization is continued as long as necessary to lyse the cells and release cytoplasmic components from the nuclei. For some cell types, as many as 100, 150, or more strokes may be required. The lysate is then transferred into a 15 ml conical tube on ice, and the cell lysis procedure is repeated with the remainder of the suspension of swollen cells. Sucrose from a 2 M stock solution made in hypotonic buffer is added to the cell lysate, resulting in a final concentration of 250 mM sucrose. This solution is mixed by inversion, and the nuclei are pelleted by centrifugation at 400 x g in a swing out rotor for 10 to 40 minutes at 4°C. The supernatant is then discarded, and the pelleted nuclei are resuspended in 10-20 volumes of nuclear buffer (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose, 25 mM KCl, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF). The nuclei are sedimented and resuspended in 1-2 volumes of nuclear buffer, as described above. The freshly isolated nuclei may either be used immediately for in vitro reprogramming and nuclear transfer into recipient cells or cytoplasts as described below or stored for later use. For storage, the nuclei are diluted in nuclear buffer to a concentration of approximately 10^6/ml. Glycerol (2.4 volumes of 100% glycerol) is added and mixed well by gentle pipetting. The suspension is aliquoted

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into 100-500 µl volumes in 1.5-ml tubes on ice, immediately frozen in a methanol-dry ice bath, and stored at -80°C. Prior to use, aliquots of the nuclei are thawed on ice or at room temperature. One volume of ice cold nuclear buffer is added, and the solution is centrifuged at 1,000 x *g* for 15 minutes in a swing out rotor. The pelleted nuclei are

5 resuspended in 100-500 µl nuclear buffer and centrifuged as described above. The pelleted nuclei are then resuspended in a minimal volume of nuclear buffer and stored on ice until use.

Preparation of the reprogramming extract

10 Interphase cultured cells as harvested using standard methods and washed by centrifugation at 500 x *g* for 10 minutes in a 10 ml conical tube at 4°C. Preferably, the cells are of the desired cell type that one wishes the recipient cell or cytoplast to become. The supernatant is discarded, and the cell pellet is resuspended in a total volume of 50 ml of cold PBS. The cells are centrifuged at 500 x g for 10 minutes at 4°C. This washing

15 step is repeated, and the cell pellet is resuspended in approximately 20 volumes of ice-cold interphase cell lysis buffer (20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, 1 mM DTT, 10 µM aprotinin, 10 µM leupeptin, 10 µM pepstatin A, 10 µM soybean trypsin inhibitor, 100 µM PMSF, and optionally 20 µg/ml cytochalasin B). The cells are sedimented by centrifugation at 800 x g for 10 minutes at 4°C. The supernatant is discarded, and the cell

20 pellet is carefully resuspended in no more than one volume of interphase cell lysis buffer. The cells are incubated on ice for one hour to allow swelling of the cells. The cells are lysed by either sonication using a tip sonicator or Dounce homogenization using a glass mortar and pestle. Cell lysis is performed until at least 90% of the cells and nuclei are lysed, which may be assessed using phase contrast microscopy. The sonication time

25 required to lyse at least 90% of the cells and nuclei may vary depending on the type of cell used to prepare the extract.

The cell lysate is placed in a 1.5-ml centrifuge tube and centrifuged at 10,000 to 15,000 x *g* for 15 minutes at 4°C using a table top centrifuge. The tubes are removed

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from the centrifuge and immediately placed on ice. The supernatant is carefully collected using a 200 μl pipette tip, and the supernatant from several tubes is pooled and placed on ice. This supernatant is the "interphase cytoplasmic" or "IS15" extract. This cell extract may be aliquoted into 20 μl volumes of extract per tube on ice and immediately flash-frozen on liquid nitrogen and stored at –80°C until use. Alternatively, the cell extract is placed in an ultracentrifuge tube on ice (e.g., fitted for an SW55 Ti rotor; Beckman). If necessary, the tube is overlayed with mineral oil to the top. The extract is centrifuged at 200,000 x g for three hours at 4°C to sediment membrane vesicles contained in the IS15 extract. At the end of centrifugation, the oil is discarded. The supernatant is carefully collected, pooled if necessary, and placed in a cold 1.5 ml tube on ice. This supernatant is referred to as "IS200" or "interphase cytosolic" extract. The extract is aliquoted and frozen as described for the IS15 extract.

If desired, the extract can be enriched with additional nuclear factors. For example, nuclei can be purified from cells of the cell type from which the reprogramming extract is derived and lysed by sonication as described above. The nuclear factors are extracted by a 10-60 minute incubation in nuclear buffer containing NaCl or KCl at a concentration of 0.15-800 mM under agitation. The lysate is centrifuged to sediment unextractable components. The supernatant containing the extracted factors of interest is dialyzed to eliminate the NaCl or KCl. The dialyzed nuclear extract is aliquoted and stored frozen. This nuclear extract is added at various concentrations to the whole cell extract described above prior to adding the nuclei for reprogramming.

As an alternative to a cell extract, a reprogramming media can also be formed by adding one or more naturally-occurring or recombinant factors (e.g., nucleic acids or proteins such as DNA methyltransferases, histone deacetylases, histones, nuclear lamins, transcription factors, activators, repressors, growth factors, hormones, or cytokines) to a solution, such as a buffer. Preferably, one or more of the factors are specific for the cell type one wishes the donor cell to become.

Reprogramming of nuclei in the extract

Either freshly isolated or thawed purified nuclei are resuspended in the reprogramming media described in the previous section at a concentration of 4,000-5,000 nuclei/μl. An ATP generating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/ml creatine kinase) and 100 μM GTP are added to the interphase extract to promote active uptake of nuclear components by the exogenous nuclei. The reaction is incubated at 30°C for up to two hours. Uptake of specific nuclear components may be monitored by immunofluorescence analysis of the nuclei, as shown in Figs. 3A and 6A.

Purification of reprogrammed nuclei out of the extract

The reprogrammed nuclei are centrifuged at 1,000 x g for 10-30 minutes through a 1 M sucrose cushion prepared in nuclear buffer at 4°C. The nuclei are washed by resuspending them in 500 μl cold nuclear buffer and centrifuging at 1,000 x g for 10 minutes at 4°C. The nuclei are resuspended in nuclear buffer and held on ice until use for nuclear transfer into the cytoplasm of recipient cells or cytoplasts.

Enucleation of recipient cells

Preferably, part or all of the DNA in the recipient cell is removed or inactivated. This destruction or removal of the DNA in the recipient cell prevents the genetic material of the cell from contributing to the characteristics and function of the reprogrammed cell. One method for destroying the nucleus of the cell is exposure to ultraviolet light (Gurdon, in *Methods in Cell Biology, Xenopus Laevis: -Practical Uses in cell and Molecular Biology*, Kay and Peng, eds., Academic Press, California, volume 36:pages 299-309, 1991). Alternatively, the nucleus may be surgically removed by any standard technique (see, for example, McGrath and Solter, Science 220:1300-1319, 1983). In one possible method, a needle is placed into the cell, and the nucleus is aspirated into the inner space of the needle. The needle may then be removed from the cell without rupturing the plasma membrane (U.S. Patent Numbers 4,994,384 and 5,057,420).

40

Introduction of reprogrammed nuclei into recipient cells or cytoplasts

The nuclei are introduced into recipient cells or cytoplasts of either the desired cell type or of any other cell type using standard methods, such as microinjection or electrofusion (see, for example, U.S. Patent Numbers 4,997,384 and 5,945,577). The reconstituted cells are placed back in culture and allowed to recover, divide, and differentiate according to the reprogrammed pathway. Gene expression by the reprogrammed cells may be monitored using standard Northern analysis to measure expression of mRNA molecules, preferably mRNA molecules that are specific for the donor cell, recipient cell, or the desired cell type (Ausubel *et al.*, supra). Expression of specific mRNA molecules may also be detected using standard reverse-transcription polymerase chain reaction (RT-PCR) assays with primers designed to specifically bind an mRNA molecule of interest. Alternatively, the expression of multiple cell specific mRNA molecules may be monitored using standard DNA chip technology with cDNA arrays (Marrack *et al.*, Current Opinion in Immunology 12, 206-209, 2000; Harkin, Oncologist. 5:501-507, 2000; Pelizzari *et al.*, Nucleic Acids Res. 2;28(22):4577-4581, 2000; Marx, Science 289(5485):1670-1672, 2000). The cells may be analyzed for a reduction in expression of genes specific for the cell type of the donor cell, recipient cell, or recipient cytoplast. Additionally, cells may be assayed for an increase in expression of genes specific for the desired cell type. Examples of mRNA molecules that are indicative of reprogramming to generate a stem cell include H-19, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM-2, Oct-4, Genesis, GCNF, GDF-3, and TDGF-1. Neural cell specific mRNA molecules include, but are not limited to, NGF, NF-H, NeuN, NSE, and CD11b. For analyzing the conversion to an adipocyte cell fate, expression of mRNA molecules such as leptin, PPARλ1, PPARλ2, SREBP1C, IR, and TNFα may be monitored. IGF-1 and IR are indicative of insulin producing cells. Additionally, the cells may be analyzed for expression of particular proteins using standard Western or immunofluorescence analysis (Ausubel *et al.*, *supra*).

Examples of other characteristics of the reprogrammed cell that may be analyzed to determine whether it has been converted into the desired cell type include the size of

the cell, cell morphology, ability to grow as an adherent cell, ability to grow as an attached cell, volume of cytoplasm, and location of a centrosome. The functions of the reprogrammed cells may also be tested, such as the ability of red blood cells to transport O_2 and CO_2, the ability of B-cells to make antibodies, and the ability of neutrophiles to

5   phagocytose and destroy invading bacteria. Additionally, the production of lipids by adicotypes may be determined using standard microscopy to visualize lipid droplets in the cells.

Example 2: Two-step *in vitro* reprogramming method

10   In another method for reprogramming cells, nuclei are isolated from interphase cells and incubated in a mitotic extract, a detergent and salt solution, or a protein kinase solution to induce nuclear envelope breakdown and the formation of chromatin masses. This incubation causes the release of factors from the chromatin masses. Alternatively, chromatin masses may be isolated from mitotic cells. Preferably, the chromatin masses

15   are then incubated in an interphase reprogramming extract to promote the formation of nuclear membranes and the addition of desired factors from the extract to the resulting nuclei. The reprogrammed nuclei are then isolated from the extract and inserted into recipient cells or cytoplasts of the desired cell type or of any other cell type.

Alternatively, the chromatin masses may be directly inserted into recipient cells or

20   cytoplasts without first being induced to reform nuclei. For this embodiment, recipient cells or cytoplasts of the desired cell type are used so that desired factors from the cytoplasm of the recipient cells or cytoplasts may bind the exogenous chromosomes from the donor chromatin masses and further promote the expression of desired mRNA and protein molecules.

25

Preparation of mitotic cell extract

A mitotic cytoplasmic (MS15) or mitotic cytosolic (MS200) extract may be prepared as described above for interphase IS15 or IS200 extract, except that mitotic cells are used instead of interphase cells and that 10 mM EDTA is added to the cell lysis

buffer. If desired, the extract can be enriched with additional nuclear factors as described in Example 1. For the isolation of mitotic cells, somatic cells are synchronized in mitosis by incubating them in 0.5-1 µg/ml nocodazole for 17-20 hours, and the mitotic cells are detached by vigorous shaking, as described above. The detached G_1 phase doublets may

5   be discarded, or they may be allowed to remain with the mitotic cells which constitute the majority (over 80%) of the detached cells. The harvested detached cells are centrifuged at 500 x *g* for 10 minutes in a 10 ml conical tube at 4°C.

Chromosome condensation reaction in mitotic extract for removal of endogenous nuclear

10   components

An aliquot of MS15 or MS200 extract is thawed on ice. An ATP-generating system (0.6 µl) and GTP are added to 20 µl of extract and mixed by vortexing, resulting in final concentrations of 1 mM ATP, 10 mM creatine phosphate, 25 µg/ml creatine kinase, and 100 µM GTP.

15   Nuclei are isolated from donor cells as described above. The nuclei suspension is added to the extract at a concentration of 1 µl nuclei per 10 µl of extract, mixed well by pipetting, and incubated in a 30, 33, 35, 37, or 39°C water bath. The tube containing the mixture is tapped gently at regular intervals to prevent chromosomes from clumping at the bottom of the tube. Nuclear envelope breakdown and chromosome condensation is

20   monitored at regular intervals, such as every 15 minutes, under a microscope. When the nuclear envelope has broken down and chromosomes have started to condense, the procedure for recovery of chromatin masses from the extract is started.

Formation of decondensed chromatin masses by exposure of nuclei to mitotic extract and

25   anti-NuMA antibodies

Alternatively, chromatin masses that are not condensed or only partially condensed may be formed by performing the above procedure after pre-loading the isolated nuclei with an antibody to the nuclear matrix protein NuMA (Steen *et al.*, J. Cell Biol. 149, 531-

536, 2000). This procedure allows the removal of nuclear components from chromatin by the dissolution of the nuclear membrane surrounding the donor nuclei; however, the condensation step is inhibited by addition of the anti-NuMA antibody. Preventing chromosome condensation may reduce the risk of chromosome breakage or loss while the chromosomes are incubated in the mitotic extract.

For this procedure, purified cell nuclei (2,000 nuclei/μl) are permeabilized in 500 μl nuclear buffer containing 0.75 μg/ml lysolecithin for 15 minutes at room temperature. Excess lysolecithin is quenched by adding 1 ml of 3% BSA made in nuclear buffer and incubating for 5 minutes on ice. The nuclei are then sedimented and washed once in nuclear buffer. The nuclei are resuspended at 2,000 nuclei/μl in 100 μl nuclear buffer containing an anti-NuMA antibody (1:40 dilution; Transduction Laboratories). After a one hour incubation on ice with gentle agitation, the nuclei are sedimented at 500 x g through 1 M sucrose for 20 minutes. The nuclei are then resuspended in nuclear buffer and added to a mitotic extract containing an ATP regenerating system, as described in the previous section. Optionally, the anti-NuMA antibody may be added to the extract to further prevent chromosome condensation.

Formation of decondensed chromatin masses by exposure of nuclei to a detergent or protein kinase

Chromatin masses that are not condensed or only partially condensed may also be formed by exposure to a detergent or protein kinase. A detergent may be used to solubilize nuclear components that are either unbound or loosely bound to the chromosomes in the nucleus, resulting in the removal of the nuclear envelope. For this procedure, purified cell nuclei (2,000-10,000 nuclei/μl) are incubated in nuclear buffer supplemented with a detergent, such as 0.1% to 0.5% Triton X-100 or NP-40. To facilitate removal of the nuclear envelope, additional salt, such as NaCl, may be added to the buffer at a concentration of approximately 0.1, 0.15, 0.25, 0.5, 0.75, or 1 M. After a 30-60 minute incubation on ice with gentle shaking, the nuclei are sedimented by

centrifugation at 1,000 x g in a swing-out rotor for 10-30 minutes, depending on the total volume. The pelleted nuclei are resuspended in 0.5 to 1 ml nuclear buffer and sedimented as described above. This washing procedure is repeated twice to ensure complete removal of the detergent and extra salt.

5   Alternatively, the nuclear envelope may be removed using recombinant or naturally-occurring protein kinases, alone or in combination. Preferably, the protein kinases are purified using standard procedures or obtained in purified form from commercial sources. These kinases may phosphorylate components of the nuclear membrane, nuclear matrix, or chromatin, resulting in removal of the nuclear envelope

10   (see, for example, Collas and Courvalin, Trends Cell Biol. 10: 5-8, 2000). Preferred kinases include cyclin-dependent kinase 1 (CDK1), protein kinase C (PKC), protein kinase A (PKA), MAP kinase, and calcium/calmodulin-dependent kinase (CamKII). For this method, approximately 20,000 purified nuclei are incubated in 20 μl of phosphorylation buffer at room temperature in a 1.5 ml centrifuge tube. A preferred

15   phosphorylation buffer for CDK1 (Upstate Biotechnology) contains 200 mM NaCl, 50 mM Tris-HCl (pH 7.2-7.6), 10 mM $MgSO_4$, 80 mM β-glycerophosphate, 5 mM EGTA, 100 μM ATP, and 1 mM DTT. For PKC, a preferred buffer contains 200 mM NaCl, 50 mM Tris-HCl (pH 7.2-7.6), 10 mM $MgSO_4$, 100 μM $CaCl_2$, 40 μg/ml phosphatidylserine, 20 μM diacylglycerol, 100 μM ATP, and 1 mM DTT. If both PKC and CDK1 are used

20   simultaneously, the CDK1 phosphorylation buffer supplemented with 40 μg/ml phosphatidylserine and 20 μM diacylglycerol is used. A preferred phosphorylation buffer for PKA includes 200 mM NaCl, 10 mM MgSO4, 10 mM Tris, pH 7.0, 1mM EDTA, and 100 μM ATP. For MAP kinase, the PKA phosphorylation buffer supplemented with 10 mM $CaCl_2$, and 1 mM DTT may be used. For CamKII, either

25   PKA buffer supplemented with 1 mM DTT or a Cam Kinase assay kit from Upstate Biotechnology (Venema et al. J. Biol. Chem 272: 28187-90, 1997) is used.

       The phosphorylation reaction is initiated by adding a protein kinase to a final amount of 25-100 ng. The reaction is incubated at room temperature for up to one hour.

Nuclear envelope breakdown may be monitored by microscopy during this incubation, such as at 15 minute intervals. After nuclear envelope breakdown, nuclei are washed three times, as described above for the removal of the detergent solution.

5 Recovery of chromatin masses from the extract, detergent and salt solution, or protein kinase solution

The extract or solution containing the condensed, partially condensed, or not condensed chromatin masses is placed under an equal volume of 1 M sucrose solution made in nuclear buffer. The chromatin masses are sedimented by centrifugation at 1,000 10 x g for 10-30 minutes depending on the sample volume in a swing out rotor at 4°C. The supernatant is discarded and the pelleted chromatin masses are carefully resuspended by pipetting in 0.1-1.0 ml nuclear buffer and centrifuged at 1,000 x g for 10-30 minutes. The supernatant is discarded, and the pelleted chromatin masses are resuspended in nuclear buffer and stored on ice until use.

15

Isolation of chromatin masses from mitotic cells

As an alternative to generating chromatin masses by exposure of nuclei to a mitotic extract, a detergent and salt solution, or a protein kinase solution, chromatin masses may be obtained by lysis of cells synchronized in mitosis and centrifugation of 20 the cell lysate as described herein.

Preparation of membrane vesicles for nuclear reassembly *in vitro*

The pellet generated from the 200,000 x g centrifugation during the preparation of the MS2000 mitotic extract is used as a source of mitotic membrane vesicles. This pellet 25 is resuspended in membrane wash buffer (250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 50 mM Hepes, pH 7.5, 1 mM DTT, 1 mM ATP, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF), centrifuged at 100,000 x g for 30 minutes, aliquoted, frozen in liquid nitrogen, and stored at -80°C.

Nuclear reassembly assay

If desired, nuclei may be reassembled from condensed, partially condensed, or decondensed chromatin masses as described below. The reformation of the nuclear membrane around the chromosomes may encapsulate factors from the extract used for reassembly allowing them to be transferred as part of the reformed nucleus into the recipient cell or cytoplast. The chromatin masses are recovered by sedimentation through a 1 M sucrose cushion and are resuspended in interphase extract at a concentration of 4,000-5,000 chromatin masses/μl. Preferably, this interphase extract is formed from cells of the cell type that is desired, as described above. The extract is supplemented with membrane vesicles prepared as described above to provide membranes which are required for nuclear envelope assembly. The membranes are added at a concentration of 1 μl thawed membranes per 10 μl extract and mixed by vortexing. An ATP generating system (2 mM ATP, 20 mM creatine phosphate, 50 μg/ml creatine kinase) and 100 μM GTP are added to the interphase extract to promote chromatin decondensation, binding of nuclear membrane vesicles to chromatin, and vesicle fusion to form an intact nuclear membrane. The reaction is incubated at 30°C for up to two hours, and nuclear reassembly is monitored by phase contract microscopy.

Purification of reprogrammed nuclei out of the extract

Reprogrammed nuclei are centrifuged at 1,000 x g for 10-30 minutes through a 1 M sucrose cushion prepared in nuclear buffer at 4°C. The nuclei are washed by resuspending them in 500 μl cold nuclear buffer and sedimentation at 1,000 x g for 10 minutes at 4°C. Then, the nuclei are resuspended in nuclear buffer and held on ice until use for nuclear transfer into the cytoplasm of recipient cells or cytoplasts.

Introduction of reprogrammed nuclei or chromatin masses into recipient cells or cytoplasts

The chromatin masses or nuclei formed from the chromatin masses are inserted into recipient cells or cytoplasts using standard methods, and gene expression is monitored, as described above.

Example 3: Reprogramming of permeabilized cells without nuclear transfer

Cells may also be reprogrammed without requiring the isolation of nuclei or chromatin masses from the cells. In this method, interphase or mitotic cells are permeabilized and then incubated in an interphase or mitotic reprogramming extract under conditions that allow the exchange of factors between the extract and the cells. If an interphase extract is used, the nuclei in the cells remain membrane-bounded; if a mitotic extract is used, nuclear envelope breakdown and chromatin condensation may occur. After the nuclei are reprogrammed by incubation in this extract, the plasma membrane is preferably resealed, forming an intact reprogrammed cell that contains desired factors from the extract. If desired, the extract can be enriched with additional nuclear factors as described in Example 1.

Permeabilization of cells

Cells that may be reprogrammed using this procedure include unsynchronized cells and cells synchronized in G_o, G_1, S, G_2, or M phase or a combination of these phases. The cells are permeabilized using any standard procedure, such as permeabilization with digitonin or Streptolysin O. Briefly, cells are harvested using standard procedures and washed with PBS. For digitonin permeabilization, cells are resuspended in culture medium containing digitonin at a concentration of approximately 0.001 – 0.1% and incubated on ice for 10 minutes. For permeabilization with Streptolysin O, cells are incubated in Streptolysin O solution (see, for example, Maghazachi *et al.*, 1997 and references therein) for 15-30 minutes at room temperature. After either incubation, the cells are washed by centrifugation at 400 x *g* for 10 minutes.

This washing step is repeated twice by resuspension and sedimentation in PBS. Cells are kept in PBS at room temperature until use. Alternatively, the cells can be permeabilized while placed on coverslips as described in Example 6 to minimize the handling of the cells and to eliminate the centrifugation of the cells, thereby maximizing the viability of the cells. Preferably, the cells are immediately added to the interphase or mitotic extract for reprogramming, as described below.

Reprogramming of cells in extract

An interphase or mitotic extract is prepared as described above, preferably using cells of the cell type that one desires the permeabilized cells to become. The permeabilized cells are suspended in the reprogramming extract at a concentration of approximately 100-1,000 cells/μl. The ATP generating system and GTP are added to the extract as described above, and the reaction is incubated at 30-37°C for up to two hours to promote translocation of factors from the extract into the cell and active nuclear uptake or chromosome-binding of factors. The reprogrammed cells are centrifuged at 800 x g, washed by resuspension, and centrifugation at 400 x g in PBS. The cells are resuspended in culture medium containing 20-30% fetal calf serum (FCS) and incubated for 1-3 hours at 37°C in a regular cell culture incubator to allow resealing of the cell membrane. The cells are then washed in regular warm culture medium (10% FCS) and cultured further using standard culturing conditions.

Example 4: Reprogramming using an activated T-cell extract

This reprogramming study using an activated T-cell extract is based on functional differences between resting and activated T-cells. Antigen-mediated activation of resting peripheral blood T-cells by stimulation of the T-cell antigen receptor-CD3 (TCR-CD3) complex and the CD28 co-stimulatory receptor induces chromatin remodeling and activation of numerous genes. One such gene is the T-cell-specific growth factor *interleukin-2 (IL-2)* gene. *IL-2* regulation involves the stimulation-dependent activators

NFAT, NFκB, AP-1, the constitutive transcription factor Oct-1, and the mitogen-activated protein kinase, Erk.

Cell extracts from activated human T-cells were used to induce nuclear localization of transcription factors in unactivated human T-cells, B-cells, human fibroblasts, and HeLa cells. Additionally, this incubation promoted DNA-binding of the chromatin remodeling SWI/SNF complex, hyperacetylation of the IL-2 gene and promoter, and expression of IL-2 mRNA in unactivated T-cells. Expression of IL-2 was also induced in primary vascular endothelial cells, epithelial cells, and neuronal precursor cells.

To demonstrate that activation of intact T-cells induces expression of IL-2, human T-cells were purified from peripheral blood, cultured overnight, and stimulated with anti-CD3 antibodies (clone SpvT3d obtained from A.M. Rasmussen, Norwegian Radium Hospital, Montebello, Norway). In particular, T-cells were purified from peripheral blood from healthy donors (Skalhegg *et al.*, Science 263:84-87, 1994). Cells were cultured for 20 hours and incubated on ice for 15 minutes at $5-10 \times 10^7$ cells/ml in RPMI1640 (Gibco BRL). The TCR-CD3 complex was stimulated with 5 µg/ml anti-CD3 antibodies, and the cells were incubated on ice for 30 minutes. Cells were spun at 400 x g at 4°C for 7 minutes, washed, and resuspended to 5×10^7 cells/ml in ice-cold RPMI1600. An anti-mouse Fab fragment (10 µg/ml) was added as a cross-linker, and the cells were incubated at 37°C (t=0 min post-stimulation). At the indicated time points, cells were diluted with ice-cold PBS, snap-frozen in liquid nitrogen, thawed, and washed in PBS. Total RNA was isolated, and RT-PCR was performed using IL-2-specific primers. As illustrated in Fig. 1, IL-2 mRNA is expressed in activated T-cells but not expressed in mock-treated T-cells ("30c," "60c," and "120c" denote mock-treated cells).

To determine whether extract from activated T-cells can increase nuclear localization of transcription factors in other cells, T-cells were activated by incubation in the presence of an anti-CD3 antibody (Skalhegg, *et al.*, Science 263:84-87, 1994), and then the cells were washed to remove the unbound antibody. Stimulated T-cell extracts (SE) were prepared 5-10 minutes after anti-CD3 stimulation (*i.e.*, 2 hours before onset of

50

transcription of the *IL-2* gene). This timing for preparation of the extract allowed

unequivocal detection of *IL-2* transcription in the reprogrammed nuclei because the

stimulated extract did not contain any endogenous IL-2 mRNA. To halt all reactions,

cells were snap-frozen in liquid nitrogen at 5-10 minutes post-stimulation, thawed,

5   washed in ice-cold PBS and in lysis buffer (10 mM Hepes, pH 8.2, 50 mM NaCl, 5 mM

$MgCl_2$, 1 mM DTT, and protease inhibitors), and sedimented. The pellets were

resuspended in two volumes of lysis buffer.

A stimulated T-cell extract was prepared by lysing these T-cells, centrifuging them

at 15,000 x g, isolating the supernatant, and adding an ATP-generating system to the

10   supernatant. In particular, cells were disrupted with a tip sonicator until over 90% of the

cells and nuclei were lysed. The lysate was cleared at 15,000 x g for 15 minutes at 4°C.

The supernatant was used fresh or aliquoted, frozen in liquid nitrogen, and stored at -

80°C. This simple method does not require dialysis, and therefore the extract remains

concentrated (~25 mg/ml protein), and the procedure minimizes risks of proteolysis.

15   Extract from unstimulated T-cells (USE) were prepared from mock (H_2O)-stimulated T-

cells. A reprogramming reaction consisted of 20 µl or multiples thereof of stimulated

extract or unstimulated extract containing 10^5 nuclei and an ATP generating system (1

mM ATP, 10 mM creatine phosphate, 25 µg/ml creatine kinase, and 100 µM GTP).

To generate donor nuclei, resting peripheral blood T-cells were washed and

20   resuspended in 20 volumes of ice-cold hypotonic nuclear buffer (10 mM Hepes, pH 7.5,

2 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, and protease inhibitors). Nuclei were isolated by

careful Dounce-homogenization, sedimented at 400 x g and washed in nuclear buffer

(hypotonic nuclear buffer/250 mM sucrose). HUVEC, HeLa, and NT2 nuclei were

isolated similarly. Nuclear integrity prior to, and after, incubation in the extract was

25   monitored by phase contrast microscopy and by nuclear membrane labeling with 10

µg/ml of the lipophilic dye, $DiOC_6$ (Fig. 8A). Nuclei purified from resting T-cells, from

the B-cell line Reh, 293T fibroblasts, or HeLa cells were incubated in this extract for 30

minutes at a concentration of approximately 5,000 nuclei per µl of extract and at 30°C

unless otherwise indicated. Then, the nuclei were purified by sedimentation for 10 minutes through a 1 M sucrose cushion. Alternatively, RNA was extracted from the reaction mix for RT-PCR. As demonstrated by immunofluorescence analysis, the T-cell specific transcription factor NFAT was imported into the nuclei exposed to the stimulated extract (Figs. 3A and 6A).

5

The ability of other transcription factors from the extract to migrate into the nuclei of T-cells, B-cells, fibroblasts, and HeLa cells was also determined. For this assay, input donor nuclei ("Input") from unstimulated T-cells were incubated in either stimulated extract ("SE"), control extract prepared from unstimulated T-cells (denoted "USE" for unstimulated extract), or stimulated extract containing a monoclonal antibody against nucleoporins which sterically blocks nuclear import ("SE + mAb414"). The nuclei were then purified from the extract by centrifugation and resuspension. As expected for a whole cell extract, NFAT, AP-1, or NFκB, Oct-1 and Erk (1 and 2) were detected on Western blots of input stimulated extract prior to incubation of the nuclei (Fig. 8B).

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15 Virtually no AP-1 was seen in input unstimulated extract likely because the complex is not assembled in unstimulated T-cells, and no NFAT, AP-1, NFκB and little Erk were detected in input nuclei (Fig. 8B). As illustrated in Fig. 3B and 8B, T-cell nuclei incubated in the stimulated extract had increased levels of NFAT, c-Jun/AP1, NFκB, and MAP kinase (Erk1 and Erk2), as measured using standard Western blot analysis with an

20 anti-histone H4 antibody as a loading control and with antibodies to each factor (e.g., the anti-Erk antibody obtained from Dr J. Kubiak, CNRS, Paris, France). The AP-1 transcription complex was also assembled in the nuclei (Fig. 8B), presumably as a result of Jun-Fos association. And Erk was imported into nuclei exposed to stimulated extract (Fig. 8B). Nuclear import of all factors was verified by immunofluorescence analysis.

25 Nuclear uptake of these factors in resting T-cell nuclei occurred actively through nuclear pore complexes because import was inhibited by substituting ATP or GTP with ATPγS, AMP-PNP, or GTPγS in the extract, or by functional inhibition of nuclear pores with mAb414, an antibody against several nucleoporins obtained from M. Rout, Rockefeller

University, New York (Fig. 8B, SE+414) (Davies and Blobel, Cell 45:699-709, 1986). The ubiquitous transcription factor Oct-1 was detected in similar amounts in input nuclei and nuclei exposed to stimulated extract or unstimulated extract (Fig. 8B, Oct-1). Incubation of the nuclei in the unstimulated extract had negligible effect on the level of

5   these transcription factors.

Additionally, NFAT, c-Jun/AP1, and NFκB levels were increased in nuclei from Reh B-cells, 293T fibroblasts, and HeLa cells after incubation in the stimulated T-cell extract (Fig. 6A). For example, immunological analyses of purified 293T fibroblast nuclei showed that the stimulated T-cell extract, but not the control unstimulated T-cell

10   extract, supported nuclear uptake of NFAT, NFκB, and assembly of the AP-1 transcription complex (Figs. 11A and 11B). Notably, the unstimulated extract supported nuclear import of BSA conjugated to nuclear localization signals in resting T-cell nuclei and fibroblast nuclei to the same extent as the stimulated extract (Fig. 8B BSA-NLS, and 11B), demonstrating specificity of import and assembly of transcriptional activators of

15   the *IL-2* gene for the stimulated extract.

For T-cell nuclei exposed to either the stimulated or unstimulated extract, DNA-binding by these transcription factors was assessed using a standard nuclear retention assay. This assay involves extraction of nuclei with 0.1% Triton X-100 to dissolve the nuclear membrane and sedimentation at 15,000 x g or extraction with 0.5% Triton X-100

20   for one hour and sedimentation at 10,000 x g for 10 minutes (Zhao *et al.*, Cell 95:625-636, 1998). Soluble chromatin fractions were prepared from purified nuclei by micrococcal nuclease digestion and EDTA extraction (O'Neill and Turner, Methods Enzymol. 274:189-197, 1996). Nuclear matrices, defined as Triton X-100, DNAse, and RNAse extraction-resistant structures were isolated as described previously (Steen *et al.*,

25   J. Cell Biol. 149:531-536, 2000). Immunoblot analysis was performed on the pellet, which contains transcription factors that are bound to DNA, and the supernatant, which contains the unbound transcription factors. In particular, insoluble material was dissolved in SDS, and proteins in the soluble fraction were precipitated and dissolved in SDS. Equal protein amounts of both fractions (30 μg) were analyzed by immunoblotting.

The percentage of DNA-bound transcription factors were determined by densitometric analysis of duplicate blots. The data is reported as the mean ± the standard deviation (Fig. 3C).

The results of this nuclear retention assay also support the increased nuclear
5   import and DNA-binding of NFAT, c-Jun/AP1, NFκB, and MAP kinase transcription factors in reprogrammed T-cell nuclei. For example, an increase of up to 8.5-fold in intranuclear bound NFAT, AP-1, and NFκB was detected in nuclei exposed to the stimulated extract compared to nuclei exposed to the unstimulated extract (Fig. 8C). Bound Oct-1 was detected in nuclei exposed to unstimulated extract or stimulated extract
10   (Fig. 8C) consistent with its DNA-binding ability in T-cell and non-T-cell nuclei. A two-fold increase in bound Erk also occurred in nuclei exposed to stimulated extract (Fig. 8C). Immunoblotting of soluble chromatin and nuclear matrix fractions prepared from nuclei exposed to stimulated extract indicated that NFAT, AP-1, NFκB, and Oct-1 were primarily bound to chromatin, whereas most of insoluble Erk was associated with the
15   matrix (Fig. 8D).

For demonstration of the effect of exposing T-cell nuclei to the stimulated extract on the DNA-binding of the chromatin remodeling SWI/SNF complex, resting T-cell nuclei were incubated in cell lysis buffer, the unstimulated extract, or the stimulated extract, each containing an ATP-generating system, for 30 minutes and sedimented
20   through sucrose. The percentage of DNA-bound and unbound human SWI/SNF after various incubation times was assessed using the above nuclear retention assay with anti-BRG1 antibodies to visualize the SWI/SNF complex (Figs. 4B, 4C, 9A) (Collas et al., J. Cell Biol. 147:1167-1180, 1999). In particular, BRG1 was immunoprecipitated from micrococcal nuclease-soluble chromatin pre-cleared with rabbit IgGs using a 1:40
25   dilution of anti-BRG1 antibodies for 2.5 hours. The immune complex was precipitated using protein A-sepharose beads, washed in immunoprecipitation buffer, and dissolved in SDS sample buffer (Collas et al., J. Cell Biol. 147:1167-1180, 1999). Exposure of the nuclei to the stimulated extract increased the amount of DNA-bound SWI/SNF,

suggesting that reprogramming of the nuclei was occurring. For example, densitometric analysis of blots using antibodies to BRG1, a marker of the SWI/SNF complex (Zhao *et al.*, Cell 95:625-636, 1998), showed that over 80% of SWI/SNF was in an insoluble (bound) form in nuclei exposed to the stimulated extract, while SWI/SNF remained

5   mostly soluble in input nuclei or nuclei exposed to unstimulated extract (Figs. 9A and 9B). Intranuclear binding of SWI/SNF took place within 30 minutes (Fig. 9B). The physiological relevance of SWI/SNF binding *in vitro* was illustrated by intranuclear anchoring of SWI/SNF within 30 minutes of anti-CD3 stimulation of human peripheral blood T-cells (Fig. 9C).

10   The potential activity of SWI/SNF was evaluated by measuring its relative ATPase activity in input nuclei and nuclei exposed to stimulated extract or unstimulated extract. Similar amounts of SWI/SNF were immunoprecipitated from purified nuclear lysates using anti-BRG1 antibodies (Fig. 9D). Hydrolysis of 1 nM exogenous ATP by each immune precipitate ("BRG1-IP") was determined in a luciferin-luciferase assay.

15   Control precipitates using pre-immune IgGs were used as a reference (Fig. 9D, Pre-I IgG). ATPase activity was expressed as relative light units in the assay after subtraction of the pre-immune IgG reference value of 2,700. BRG1-IP purified from input nuclei or nuclei exposed to unstimulated extract displayed no or little ATPase activity. However, BRG1-IP isolated from nuclei exposed to stimulated extract showed an 8-fold increase in

20   ATPase activity compared to input nuclei (Fig. 9D). Furthermore, stimulated extract-induced ATPase activity was reduced close to basal levels when BRG1-IP was treated with DNAse I prior to the assay (Fig. 9D). These results indicate that intranuclear bound SWI/SNF complex exhibits DNA-dependent ATPase activity specific for the stimulated T-cell extract.

25   To measure hyperacetylation of the IL-2 gene of T-cell nuclei *in vivo* and *in vitro*, micrococcal nuclease was used to digest the chromatin from resting T-cells, anti-CD3-stimulated T-cells, T-cell nuclei exposed to an unstimulated extract, and T-cell nuclei exposed to a stimulated extract, forming soluble chromatin fragments. Acetylated histone H4 ("H4ac") was immunoprecipitated from the soluble chromatin fraction, and

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DNA was isolated from immune precipitate ("bound") and supernatant ("unbound") fractions. The DNA was dot-blotted on duplicate Hybon N filters and hybridized to either a fluoresceinated IL-2 probe to the IL-2 coding region or a carp β-actin probe. Hybridization was detected using alkaline phosphatase-conjugated anti-fluorescein

5   antibodies. Hyperacetylation of the IL-2 gene, but not the control β-actin gene, was observed in nuclei exposed to the stimulated extract, further suggesting that the nuclei were being reprogrammed to express genes usually repressed by the nuclei.

Acetylation of histone H4 in the *IL-2* promoter after T-cell stimulation in culture and in quiescent T-cell nuclei exposed to unstimulated extract or stimulated extract was

10   also measured by chromatin immunoprecipitation analysis, using anti-H4 and H4ac antibodies from Serotec. Hyperacetylation of the *IL-2* promoter was examined by chromatin immunoprecipitation from mock (H$_2$O) and anti-CD3-stimulated T-cells after solubilization with 0.1 U microccocal nuclease per μg DNA to from mono- and di-nucleosomes. An anti-pan-acetylated histone H4 (acH4) antibody was used to detect

15   acetylated histones (O'Neill and Turner, Methods Enzymol. 274:189-197, 1996). DNA was isolated by phenol-chloroform extraction from input, antibody-bound, and unbound chromatin fractions, and *IL-2* was identified by dot blot analysis using an *IL-2* probe. The *IL-2* promoter probe was synthesized by random prime labeling with fluoresceinated nucleotides (Gene Images CDP-Star, Amersham), using as the template a cloned 430

20   base pair insert corresponding to the 360 base pairs of the promoter/enhancer regions proximal to the start site and the first 70 base pairs of the IL-2 coding region (exon I). Primers used were 5'-GCTATTCACATGTTCAGTGTAG-3' (SEQ ID NO: 1) to hybridize the promoter region and 5'-GACAGGAGTTGCATCCTGTACA-3' (SEQ ID NO: 2) to hybridize to exon I. The β-actin probe was synthesized as described above

25   using a cloned *Sal*I-*Nco*I 1.3-kb insert of β-actin intron I as a template (Collas *et al.*, J. Cell Sci. 112:1045-1054, 1999b). Hybridization was detected by chemiluminescence using alkaline phosphatase-conjugated anti-fluorescein antibodies (Collas *et al.*, J. Cell Sci. 112:1045-1054, 1999b).

In unstimulated T-cells, the *IL-2* promoter was entirely detected in the anti-acH4 unbound fraction, suggesting hypoacetylation or absence of H4 acetylation of the *IL-2* promoter (Fig. 9E, "Culture"). T-cell stimulation, however, elicited hyperactetylation of the *IL-2* promoter, as demonstrated by its high enrichment in the anti-acH4-bound

5   fraction (Fig. 9E, "Culture"). Significantly, the *IL-2* promoter was also highly enriched in H4 hyperacetylated chromatin after incubation of nuclei in stimulated extract, but not in unstimulated extract (Fig. 9E, "*In vitro*"). These results are in agreement with reports showing that chromatin configuration changes occurring in the *IL-2* promoter upon T-cell activation are confined to the minimal enhancer region from –300 base pairs to the

10   transcription start codon (Ward *et al.*, Nucleic. Acids. Res. 26:2923-2934, 1998; Rao *et al.*, J. Immunol. 167:4494-4503, 2001). Altogether, these data provide strong evidence for chromatin remodeling of the *IL-2* proximal promoter region in resting T-cell nuclei exposed to the stimulated T-cell extract.

       To demonstrate the ability of the stimulated extract to induce expression of IL-2,

15   resting T-cell nuclei were incubated for 30 minutes at 30°C in unstimulated extract or stimulated extract. As controls, nuclei were incubated in stimulated extract containing either 100 µg/ml RNAse A, 100 µg/ml DNAse I, mAb414 antibodies, or the lectin WGA. After 30 minutes at 30°C, nuclei were lysed in the extracts by sonication and 3 µl extract aliquots were removed for RT-PCR analysis using IL-2-specific primers. In particular,

20   total RNA was isolated using the Qiagen RNeasy kit, and 15 ng RNA was used as the template for RT-PCR using the Promega Access RT-PCR System. A 467-bp IL-2 cDNA was amplified using the IL-2-specific primers 5'-ATGTACAGGATGCAACTCCTGTCTT-3' (SEQ ID NO: 3) and 5'-GTTAGTGTTGAGATGATGCTTTGAC-3' (SEQ ID NO:4). PCR conditions were 30

25   cycles of denaturation at 94°C for one minute, annealing at 60°C for two minutes, and extension at 72°C for three minutes. Input stimulated extract and a control stimulated extract containing 1.2 µg total RNA isolated from IL-2-producing T-cells were also analyzed (Fig. 5A and 10A). These results indicate that IL-2 expression was induced by incubation of T-cell nuclei in the stimulated extract but not by incubation in any of the

control extracts. The PCR product of the expected size (467 base pairs) was absent from input nuclei and input stimulated extract (as expected from Fig. 7), nuclei exposed to unstimulated extract (Nuclei-USE), and nuclei exposed to stimulated extract followed by treatment with 100 µg/ml RNAse A, but not 100 µg/ml DNAse I, prior to RT-PCR.

5   Thus, detection of IL-2 mRNA was the result of *IL-2* transcription and not of RNA contamination in input nuclei or in the extract. *IL-2* transcription required active nuclear import because IL-2 transcription was abolished when nuclear pore function was blocked in the stimulated extract with the mAb414 antibody or 0.5 mg/ml wheat germ agglutinin (Fig. 10A).

10   As illustrated in Figs, 5B and 10B, this *in vitro* IL-2 mRNA production is dependent on PolII transcription. For this assay, nuclei were exposed for 30 minutes to stimulated extract containing increasing concentrations of the RNA PolII inhibitor actinomycin D (0, 5, 10, 50, 100 and 500 nM), and IL-2 mRNA synthesis was analyzed by RT-PCR. As a control, extracts from anti-CD3 stimulated B-cells, fibroblasts, and

15   HeLa cells, which do not express IL-2, were tested for their ability to induce IL-2 expression in nuclei from resting T-cells. As expected, these extract failed to induce IL-2 expression. Arrows in Figs. 5A-5C point to the 458 base pair IL-2 RT-PCR product.

As a more stringent indicator of nuclear reprogramming, activation of the *IL-2* gene was monitored in nuclei purified from primary human umbilical vein endothelial

20   cells (HUVEC), NT2 neuronal precursors, and HeLa cells after a two hour incubation in the stimulated extract. RT-PCR analysis indicated that the stimulated extract activated the *IL-2* gene in the nuclei of all of these cell types; in contrast, the unstimulated extract was ineffective at inducing IL-2 transcription (Fig. 10C). *IL-2* activation was dependent on RNA Pol II activity and nuclear import, based on its elimination by 50 nM

25   actinomycin D and mAb414, respectively (Fig. 10C). Lastly, the specificity of *IL-2* induction for the stimulated extract was demonstrated by *IL-2* remaining repressed in resting T-cell nuclei exposed to control extracts from 293T fibroblasts, HeLa endothelial cells, or Bjab B-cells that had been treated with anti-CD3 and cross-linking antibodies to mimic T-cell stimulation (Fig. 10D).

In summary, these results demonstrate that nuclear reprogramming, as evidenced by transcriptional activation of a silent gene, can be induced in purified intact nuclei. Expression of the *IL-2* gene was coincident with physiological nuclear uptake and assembly of transcriptional regulatory proteins. It is noteworthy that NFAT, NFκB, and

5   AP-1 are transcription factors that reflect a proliferative response rather than a differentiation response per se. Remodeling of chromatin was demonstrated by intranuclear anchoring and DNA-dependent ATP hydrolysis activity of the SWI/SNF complex. The SWI/SNF complex uses energy of ATP hydrolysis to alter nucleosomal conformation. Notably, the stimulated extract elicited an 8-fold enhancement of ATPase

10   activity over that of input resting T-cell nuclei, from equivalent amounts of immunoprecipitated BRG1. Thus, increased ATPase activity is the result of activation of SWI/SNF rather than a consequence of higher amounts of precipitated BRG1 in nuclei exposed to stimulated extract. Histone H4 hyperacetylation of the *IL-2* proximal promoter region, which involves a complex targeting acetyltransferases to their site of

15   action, further indicates active chromatin remodeling. While not meant to limit the invention to any particular mechanism, H4 acetylation in the *IL-2* promoter may stimulate binding of transcriptional activators that would otherwise be excluded from repressed chromatin, or transcription factor binding may promote alterations in chromatin structure. The results described herein indicate nuclear reprogramming can take place in

20   intact nuclei *in vitro*. The results also demonstrate that the process involves the active intranuclear assembly of protein complexes that remodel chromatin as well as the binding of transcriptional regulators.

Example 5: Reprogramming of fibroblasts using an activated T-cell extract

25   As demonstrated in Example 4, a stimulated T-cell extract increased nuclear localization of T-cell specific transcription factors in 293T fibroblasts. The ability of fibroblasts to be reprogrammed into T-cells is characterized further below.

For this study, T-cells were purified from peripheral blood from healthy donors, as described in Example 4 (Skalhegg *et al.*, Science 263:84-87, 1994). To prepare

reprogramming extracts from stimulated T-cells, cells were frozen in liquid nitrogen at 5-10 minutes post-stimulation, thawed, washed in ice-cold lysis buffer (Collas *et al.*, J. Cell Bio. 147:1167-1180, 1999), and sedimented at 400 x g. The pellets were resuspended in two volumes of lysis buffer. Cells and nuclei were disrupted with a tip sonicator, and the lysate was cleared by centrifugation at 15,000 x g for 15 minutes at 4°C. The supernatant was used immediately or was frozen in liquid nitrogen and stored at -80°C. Unstimulated T-cell extracts were prepared from mock (H_2O)-stimulated T-cells.

Nuclear reprogramming reactions consisted of 20 µl or multiples thereof of stimulated T-cell extract or unstimulated T-cell extract containing 100,000 nuclei and an ATP generating system (1 mM ATP, 10 mM creatine phosphate, 25 µg/ml creatine kinase, and 100 µM GTP). Reactions were incubated at 30°C for 30 minutes unless indicated otherwise. At the end of incubation, nuclei were purified by sedimentation through 1 M sucrose. Alternatively, total RNA was extracted from the reaction mix for RT-PCR.

The active uptake of transcription factors in fibroblast nuclei exposed to the stimulated T-cell extract was further demonstrated by the ability of a monoclonal antibody that reacts with nucleoporins and inhibits nuclear pore function (obtained from M. Rout, Rockefeller University, New York, NY, USA) to reduce nuclear import of these factors (Fig. 11B, mAb414). Oct-1 was detected in 293T input nuclei and nuclei exposed to stimulated T-cell extract or control unstimulated T-cell extract (Fig. 11B), consistent with its DNA-binding property in several cell types. Immunoblotting of chromatin and nuclear matrices of 293T nuclei treated with the stimulated T-cell extract indicated that NFAT, AP-1, NFκB, and Oct-1 were primarily bound to chromatin (Fig. 11C). Altogether, these data demonstrate physiological uptake of transcriptional regulators by the fibroblast nuclei from the extract.

Intranuclear anchoring of the human nucleosome remodeling complex SWI/SNF was also investigated. Anti-CD3 stimulation of T-cells elicited intranuclear anchoring of the SWI/SNF complex, as determined by immunoblotting of Triton X-100-soluble and

insoluble nuclear fractions with an antibody against BRG1, a marker of the SWI/SNF complex (Fig. 12A). In particular, BRG1 was immunoprecipitated from micrococcal nuclease (MNase)-soluble chromatin pre-cleared with rabbit IgGs, using anti-BRG1 antibodies (dilunstimulated T-cell extract 1:40) for 2.5 hours. The immune complex was

5   precipitated using protein A-sepharose beads, washed three times in immunoprecipitation buffer (Collas *et al.*, J. Cell Bio. 147:1167-1180, 1999), and dissolved in SDS sample buffer. Western blots were performed using antibody dilutions of 1:500 (Collas *et al.*, J. Cell Bio. 147:1167-1180, 1999). Over 80% of SWI/SNF was detected in a bound form within 20 minutes in stimulated T-cell extract-treated 293T nuclei; in contrast, SWI/SNF

10   remained soluble in nuclei exposed to unstimulated T-cell extract (Fig. 12A).

Additionally, ATPase activity of the SWI/SNF complex was determined in a standard luciferin-luciferase assay after immunoprecipitation of the complex using anti-BRG1 antibodies. BRG1 immune precipitates ("BRG1-IPs") purified from 293T input nuclei or unstimulated T-cell extract-treated nuclei displayed no or little ATPase, based

15   on elevated ATP levels in the assay (Fig. 12B). However, BRG1-IP isolated from nuclei treated with the stimulated T-cell extract displayed an ~8-fold increase in ATPase activity, reducing the ATP level in the assay from 2,500 to 300 RLU. No activity was detected in control precipitates using pre-immune IgGs (Fig. 12B). These results indicate that the stimulated T-cell extract promotes intranuclear anchoring of the SWI/SNF

20   nucleosome remodeling complex and ATPase activity of the bound complex in 293T nuclei.

Potential for gene expression often correlates with hyperacetylation of histone H4. As an additional marker of nuclear reprogramming, changes in H4 acetylation at the *IL-2* locus in stimulated T-cell extract- and unstimulated T-cell extract-treated 293T nuclei

25   were measured. Chromatin immunoprecipitation (ChIP) experiments were performed using an antibody against all forms of acetylated H4 ("acH4," Fig. 12C). In particular, intact nuclei were isolated from 293T, NT2, and unstimulated peripheral blood T-cells by Dounce-homogenization and stored frozen (Collas *et al.*, J. Cell Bio. 147:1167-1180, 1999). Soluble chromatin was prepared from purified nuclei by MNase digestion

(O'Neill and Turner, Methods Enzymol. 274:189-197, 1996), and nuclear matrices, defined as Triton X-100, DNAse, and RNAse extraction-resistant structures, were isolated as described (Steen *et al.*, J. Cell Biol. 149:531-536, 2000). ChIP was performed after solubilization of chromatin with 0.1 U MNase per μg DNA using an anti-pan-

5   acetylated histone H4 antibody (O'Neill and Turner, Methods Enzymol. 274:189-197, 1996). DNA was isolated by phenol-chloroform extraction from antibody-bound and unbound fractions, and the *IL-2* locus was identified by dot blot analysis. An *IL-2* probe was synthesized and fluoresceinated by random priming (Amersham) using as a template a 467-bp IL-2 PCR product amplified from genomic DNA by PCR as described above.

10   Hybridization was detected by chemiluminescence (Collas *et al.*, J. Cell Sci. 112:1045-1054, 1999). The β-actin probe was synthesized and labeled as described (Collas *et al.*, J. Cell Sci. 112:1045-1054, 1999).

In input nuclei and nuclei incubated in unstimulated T-cell extract, IL-2 was detected exclusively in anti-acH4 unbound chromatin, suggesting hypoacetylation of H4

15   in the IL-2 locus (Fig. 12C). In contrast, IL-2 was detected in anti-acH4 bound chromatin of nuclei treated with stimulated T-cell extract, reflecting enhanced H4 acetylation at the IL-2 locus in these nuclei (Fig. 12C). As anticipated, reprobing filters with a probe against the constitutively expressed β-actin gene revealed hyperacetylated H4 at the β-actin locus (Fig. 12C). Thus, stimulated T-cell extract elicits enhanced H4 acetylation at

20   the IL-2 locus in 293T nuclei, providing evidence for physiological chromatin remodeling.

Another and more stringent indicator of nuclear reprogramming was induction of IL-2 transcription in 293T fibroblast nuclei exposed to stimulated T-cell extract (Fig. 12D). Total RNA was isolated using the Qiagen RNeasy kit, and 15 ng RNA was used as

25   the template for RT-PCR using the Promega Access RT-PCR System. A 467-bp product was amplified using the IL-2-specific primers 5'-ATGTACAGGATGCAACTCCTGTCTT-3' (SEQ ID NO: 5) and 5'-GTTAGTGTTGAGATGATGCTTTGAC-3' (SEQ ID NO: 6) by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for one minute, and extension at

72°C for one minute. RT-PCR analysis indicated that the IL-2 gene was activated in the stimulated T-cell extract, but not in the unstimulated T-cell extract. As expected from the above results, the IL-2 transcript was absent from input nuclei, input stimulated T-cell extract, and nuclei exposed to stimulated T-cell extract containing either 100 µg/ml

5   RNAse A, mAb414, or 50 nM of the RNA polymerase II (Pol II) inhibitor, actinomycin D (Fig. 12D). Similar results were obtained with neuronal precursor NT2 nuclei and resting T-cell nuclei (Fig. 12D). Collectively, these data indicate that the stimulated T-cell extract supports chromatin remodeling and RNA Pol II-dependent activation of the repressed IL-2 gene in T-cell and non-T-cell nuclei.

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Example 6: Reprogramming of permeabilized cells

    The ability to reprogram whole cells, in addition to purified nuclei, was demonstrated as described below. 293T fibroblasts grown on coverslips were reversibly permeabilized with the bacterial toxin Streptolysin O, exposed to extract of readily

15   available stimulated Jurkat cells or neuronal precursor cells, resealed with 2 mM $CaCl_2$, and expanded in culture. Reprogramming into T-cells was evaluated by alterations in gene expression, expression of T-cell-specific proteins, and induction of a T-cell-specific function in the reprogrammed 293T fibroblasts. Reprogrammed 293T fibroblasts exposed to neuronal extracts were analyzed for expression of neuronal proteins.

20   For these studies, 293T fibroblasts were grown on 16-mm poly-L-lysine-coated coverslips in RPMI1640 to 100,000 cells/coverslip in 12-well plates. Cells were permeabilized in 200 ng/ml streptolysin O in Ca^{2+}-free Hanks Balanced Salt Solution (Gibco-BRL) for 50 minutes at 37°C in regular atmosphere. Over 80% of 293T-cells were permeabilized under these conditions, as judged by propidium iodide uptake.

25   Streptolysin O was aspirated; coverslips overlaid with 80 µl of either 293T, Jurkat-Tag, or NT2 extract; and incubated for one hour at 37 °C in CO_2 atmosphere. Each extract contained the ATP generating system and 1 mM each of ATP, CTP, GTP and UTP. Extracts from Jurkat-TAg cells were prepared as described above after co-stimulation for

1-2 hours with 40 ng/ml anti-CD3 antibodies (clone SpvT3d obtained from A.M.

Rasmussen, Norwegian Radium Hospital, Montebello, Norway) and 0.1 μM PMA. The

neuronal precursor NT2 extract was prepared from confluent NT2 cells (Stratagene) by

sonication and sedimentation as described above. To reseal plasma membranes,

5   RPMI1640 containing 2 mM $CaCl_2$ (added from a 1 M stock in H_2O) was added to the

wells, and the cells were incubated for two hours at 37 °C. This procedure resealed

~100% of the permeabilized cells. Ca^{2+}-containing RPMI was replaced by RPMI, and

the cells were expanded for several weeks.

Transcription levels in reprogrammed fibroblasts exposed to the Jurkat extract

10   were compared to those of 293T-cells exposed to a 293T extract ('control cells') 10 days

post-reprogramming reaction. A human cytokine expression array containing 375

cDNAs was used to monitor changes in gene expression. In particular, mRNA was

isolated (mRNA Direct™, Dynal) from 'reprogrammed' and control cell pellets frozen in

liquid nitrogen at 10 days post-reprogramming. One μg mRNA was used as the template

15   for cDNA synthesis (cDNA Labeling and Hybridization Kit, R&D Systems) with α-^{33}P-

dCTP and cytokine-specific primers (R&D Systems) according to the manufacturer's

protocol. Purified probes were hybridized to Human Cytokine Expression Arrays (R&D

Systems) under recommended conditions. Arrays were exposed to a phosphorscreen for

six days. Hybridization was quantified on a phosphorimager and analyzed using the

20   Phoretix Array V.2 analysis software.

Over 120 genes were up- or down-regulated as a result of reprogramming (Fig. 13;

only transcripts up- or down-regulated more than two-fold are shown). Subsets of genes

encoding hematopoietic cell surface antigens, interleukins and interleukin receptors,

cytokines and cytokine receptors, chemokines and chemokine receptors, epidermal

25   growth factors, and orphan receptors were up-regulated. Several genes of the FGF,

adhesion molecule, and integrin families were down-regulated. Several genes of the

TGFβ and TNF families were also either up- or down-regulated. No neutrophic factor

transcripts were affected, nor was expression of house keeping genes affected (Fig. 13).

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Similar results were obtained in duplicate arrays from separate reactions examined at 13 days post-reprogramming. Thus, hematopoietic genes are turned on or up-regulated in 293T-cells exposed to a Jurkat extract, whereas genes for FGFs, adhesion molecules, and cytoskeletal components are down-regulated or repressed.

5   Expression of hematopoietic cell-specific surface antigens in reprogrammed fibroblasts was also evaluated. Immunofluorescence analysis of IL-2Rα and β was performed as described (Collas *et al.*, J. Cell Bio. 147:1167-1180, 1999; anti-IL-2Rα and IL-2Rβ antibodies were obtained from R&D Systems). Analysis of other surface antigens was performed using FITC- or TRITC-conjugated primary antibodies (FITC-

10   conjugated anti-CD3, CD4, CD8, and CD45 antibodies from Diatec and FITC-conjugated anti-TCRαβ antibody from Pharmingen). Immunofluorescence analysis showed that CD3, CD4 and CD8 were detected by 4 days post-reprogramming, and the CD45 tyrosine phosphatase was detected by 11 days post-reprogramming in most reprogrammed cells but not in control cells (Fig. 14A). Furthermore, the α and β chains of the TCR complex

15   were expressed in the reprogrammed fibroblasts, based on immunofluorescence labeling with an antibody against TCRαβ (Fig. 14B).

Expression of immune cell surface receptors in reprogrammed fibroblasts prompted investigation of functional reprogramming. Unstimulated T-cells express the low affinity IL-2 receptor β (IL-2Rβ). High affinity IL-2R requires induction of IL-2Rα by TCR-

20   CD3 complex stimulation. TCR-CD3-dependent induction of IL-2Rα is indicative of normal TCR function. Fig. 14C illustrates that reprogrammed fibroblasts expressed IL-2Rβ, but not IL-2Rα, in the absence of stimulation. Furthermore, in reprogrammed cells, anti-CD3 and phorbolmyristylacetate (PMA) stimulation elicited expression of IL-2Rα that co-localized with IL-2Rβ, as shown in overlay images (Fig. 14C, "+Stimulation").

25   Similar results were observed with the Jurkat cells that were used to prepare the extract (Fig. 14C). As expected, stimulation of control fibroblasts did not significantly induce IL-2Rα. Altogether, these results indicate the expression of functional immune-specific receptors in the reprogrammed cells.

To demonstrate the general applicability of *in vitro* cell reprogramming, permeabilized fibroblasts were exposed to an NT2 cytoplasmic and nuclear extract for one hour at 37°C as described above for Jurkat extracts. The cells were resealed and cultured for 15 days in low confluency in RPMI1640. Then, the expression of

5   neurofilament protein NF200 (Debus *et al.*, Differentiation 25:193-203, 1983) was examined by immunofluorescence. NF200 was strongly expressed in fibroblasts exposed to the NT2 extract, but not to a control 293T fibroblast extract (Fig. 15). Furthermore, NF200 appeared restricted to polarized outgrowths from the fibroblasts resembling elongating neurites, which occasionally contacted neighboring cells in culture. These

10   data indicate that neuron-specific proteins can be expressed in fibroblasts under these reprogramming conditions.

In summary, these results demonstrate functional reprogramming of a somatic cell using a nuclear and cytoplasmic extract derived from another somatic cell type. These experiments illustrate activation of repressed genes and synthesis of proteins specific for

15   another cell type in somatic fibroblasts by exposure to extracts from heterologous somatic cell types. Reprogramming fibroblast genome function in a T-cell or Jurkat-TAg extract is demonstrated by physiological nuclear uptake and assembly of transcriptional regulatory proteins, chromatin remodeling, activation of lymphoid-specific genes, down-regulation of selective sets of genes, expression of T-cell-specific antigens including CD3

20   and TCR, and establishment of the IL-2R assembly pathway in response to CD3-TCR stimulation. Moreover, a neurofilament protein was expressed in fibroblasts exposed to a neuronal precursor cell extract. *In vitro* reprogramming of differentiated somatic cells from primary cultures creates a wide range of possibilities to produce isogenic or substantially isogenic replacement cells for therapeutic applications.

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Example 7: Reprogramming to generate stem cells

An embryonic stem cells extract was used to reprogram permeabilized, mouse fibroblasts as described below. Similar methods can be used to reprogram other cells, such as other fibroblasts (*e.g.*, human skin fibroblasts).

Briefly, mouse embryonic stem cells were cultured in the presence of leukemia inhibitory factor (LIF) without feeder layers using standard procedures. An embryonic stem cell reprogramming extract was prepared as follows. Embryonic stem cells were harvested, washed three times in PBS, washed once in ice-cold cell lysis buffer described above, and the cell pellet was resuspended in an equal volume of cell lysis buffer. The suspension was sonicated on ice until all cells and nuclei were disrupted. The resulting lysate was centrifuged at 15,000 x g for 15 minutes at 4°C. The supernatant ("reprogramming extract") was either used fresh or aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until use.

Mouse NIH3T3 cells, a transformed fibroblast cell line, were grown onto 12-mm round glass coverlips coated with poly-L-lysine to a density of ~50,000 cells per coverslip. Cells were permeabilized with Streptolysin O as described above. Sreptolysin O was removed by gentle aspiration and replaced by 80 µl of mouse embryonic stem cell extract containing an ATP generating system and nucleotides. Cells were incubated in the extract for one hour at 37°C in regular atmosphere. Culture medium (500 µl) containing 2 mM CaCl$_2$ was added directly to the cells which were subsequently allowed to reseal for two hours at 37°C in a CO$_2$ incubator. CaCl$_2$-containing medium was removed and replaced by regular embryonic stem culture medium containing LIF.

Reprogrammed NIH3T3 cells were cultured and examined on day four post-reprogramming. Phase contrast microscopy analysis showed that the cells grew in clumps, forming 'colonies' resembling those formed by embryonic stem cells (compare Figs. 16A and Fig. 16B). Some of the larger colonies such as that shown on Fig. 16B lifted off the culture dish to form embryoid bodies. In contrast, control fibroblasts permeabilized with Streptolysin O and exposed to a control NIH3T3 extract did not form colonies and maintained a typical fibroblast phenotype (compare Fig. 16B with input NIH3T3 cells in Fig. 16A). Similarly, control intact (non-permeablizied) NIH3T3 cells exposed to the embryonic cell extract did not acquire the embryonic cell phenotype (Fig. 16B). Embryonic stem cell morphology of the reprogrammed cells was seen for at least 10 days in culture.

As a molecular marker of reprogramming, the reprogrammed cells were examined for the expression of Oct4, the product of the *Oct4* gene. *Oct4* expression is unique to germ cells, stem cells, preimplantation embryos, and the epiblast of the early post-implantation embryos. Therefore, *Oct4* expression represents a useful marker for

5   identification of pluri- or toti-potent cells. *Oct4* expression was monitored four days after reprogramming by immunofluorescence using a commercially available anti-Oct4 antibody (Santa Cruz Biotechnology). Fig. 17A shows a clear anti-Oct4 labeling in input embryonic stem cells. As expected, NIH3T3 fibroblasts were not labeled (Fig. 17A). NIH3T3 cells reprogrammed in the embryonic stem cell extract exhibited anti-Oct4

10   labeling; in contrast, NIH3T3 cells exposed to a control NIH3T3 extract or intact NIH3T3 cells exposed to the embryonic stem cell extract (Fig. 17A) were not labeled with anti-Oct4. This result indicates that the reprogrammed cells express the embryonic stem cell-specific transcription factor, Oct4.

Immunofluorescence observations were verified by Western blotting analysis.

15   Fig. 17B shows that while input NIH3T3 cells did not express Oct4 ("NIH"), NIH3T3 cells exposed to the embryonic stem cell extract expressed high levels of Oct4 ("NIH/ES ext."). The expression level was similar to that of embryonic stem cells used to prepare the extract (Fig. 17B, "ES"). Control NIH3T3 cells exposed to NIH3T3 cell extract did not express Oct4, as anticipated (Fig. 17B). 50,000 NIH3T3 cells and 10,000 embryonic

20   stem cells and reprogrammed cells were used in the immunoblot shown in Fig. 17B.

A novel, rapid, sensitive and semi-quantitative assay was developed to measure alkaline phosphatase, another embryonic stem cell marker, in embryonic stem cells and in the reprogrammed cells. The assay is based on spotting 1-2 µl of a Triton X-100 soluble lysate of embryonic stem cells, NIH3T3 cells exposed to NIH3T3 extract, intact NIH3T3

25   cells exposed to embryonic stem cell extract, or of any cell of choice on a dry nitrocellulose membrane or on any other appropriate solid support. The test spot is of known protein concentration or from a known cell number to allow comparison to other spots. If desired, an aliquot containing a known amount of alkaline phosphatase or having a known level of alkaline phosphatase activity can also be spotted on the filter to

form a reference spot. The membrane was wetted in Tris-buffered saline and drained. Alkaline phosphatase was detected by applying a detection solution normally designed to detect alkaline phosphatase-conjugated DNA probes on Southern blots (Alk-Phos Direct detection solution, Amersham). Alkaline phosphatase dephosphorylates a substrate

5   contained in the detection solution, resulting in light emission. The membrane was drained and exposed to film. If the test sample is applied to a solid support other than a membrane, such as a 96-well plate, than either the 96-well plate is exposed to film or, it is exposed to a CCD camara to meaure light emitted by the alkaline phosphate detection reaction. Alkaline phosphatase in the embryonic stem cell lysate, but not in the NIH3T3

10   cell lysate, resulted in the appearance of a light spot detected on the film (Fig. 18). Additionally, permeabilized NIH3T3 cells reprogrammed in embryonic stem cell extract, but not control cells, had alkaline phosphatase (Fig. 19).

The amount of alkaline phosphatase in the test spot can be determined by comparing the signal from the test spot to the signal from the reference spot or to the

15   signal from a series of reference spots with increasing levels of alkaline phosphatase (e.g., forming a standard curve). The concentration of protein or the number of cells used to derive the test spot (e.g., the units of alkaline phosphatase mg protein) can be used to extrapolate the level of alkaline phosphatase in the original test cell or sample.

The reprogrammed cells were passaged and replated on day four using standard

20   embryonic stem culture techniques, using LIF supplemented medium. Ten days after reprogramming, however, Oct4 expression levels were greatly reduced in the reprogrammed cells. The cells also lost the typical embryonic stem cell colony morphology they acquired after the reprogramming reaction. This result may be the result of either (i) transient reprogramming of the cells, i.e., the reprogramming factors

25   are diluted out as the reprogrammed cells divide and are no longer active,
(ii) spontaneous differentiation of the reprogrammed embryonic stem cells into fibroblasts, or (iii) loss of the truly reprogrammed embryonic stem cells such that contaminating non-reprogrammed fibroblasts outgrew the embryonic stem cells and remained in the culture. If desired, expression of Oct4 and other stem cell-specific

proteins may be maintained in the reprogrammed cells for a longer period of time by performing multiple rounds of reprogramming. Moreover, the permeabilized cells can be exposed to the stem cell extract for a longer period of time during each round of reprogramming. Additional nuclear factors can also be added to the stem cell extract as

5   described above to maximize reprogramming.

Collectively, these data indicate that NIH3T3 cells exposed to an embryonic stem cell extract acquire an embryonic stem cell phenotype, express Oct4 ,and express alkaline phosphatase.

10   Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

All publications mentioned in this specification are herein incorporated by

15   reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is: